İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

DIRECTED EVOLUTION OF INDUSTRIALLY IMPORTANT PROPERTIES OF FUSARIUM OXYSPORUM

Master Thesis by

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Department: Advanced Technologies

Programme: Molecular Biology-Genetics Biotechnology

JULY 2006

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YÖNLENDİRİLMİŞ EVRİM YÖNTEMİ İLE *FUSARIUM OXYSPORUM*' UN ENDÜSTRİYEL AÇIDAN ÖNEMLİ ÖZELLİKLERİNİN GELİŞTİRİLMESİ

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ABBREVIATIONS

ATP	: Adenosine triphosphate
CDW	: Cellular Dry Weight
DDG	: Diethyl 3-(3',4'-dichlorophenyl)glutarate
EMS	: Ethyl Methane Sulphonate
Hsp	: Heat Shock Protein
IME	: Inverse Metabolic Engineering
NMR	: Nuclear Magnetic Resonance
NTG	: Nitroso-methyl guanidine
PAH	: Polycyclic Aromatic Hydrocarbons
PDA	: Potato Dextrose Agar
UV	: Ultraviolet

LIST OF SYMBOLS

c	**:** Concentration
e	**:** Molar extinction coefficient
Foxy100	**:** Wild type *Fusarium oxysporum* population
Foxy101	**:** EMS mutagenized *Fusarium oxysporum* population
l	**:** Length

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DIRECTED EVOLUTION OF INDUSTRIALLY IMPORTANT PROPERTIES OF *FUSARIUM OXYSPORUM*

SUMMARY

In this study, it was aimed to improve thermotolerance of *Fusarium oxysporum* and its lipase via evolutionary engineering strategies. For this purpose, wild-type cells were firstly exposed to ethyl methane sulphonate (EMS) mutagenesis in order to create the genetic diversity. Serial stress applications, either at constant or gradually increasing levels, were performed. Survivors of a step were passed to the following step and stress selection strategy was applied over generations of populations. Stress selection was applied on either of the two different physiological states of filamentous fungus, hyphae and spore. Generations were obtained by the application of stress at either physiological state. Stress conditions were also chosen as gradually increasing heat stress and constant stress. The final populations were obtained as the result of both exposure of spores and hyphae to two different stress conditions. Last generation obtained by exposing hyphae to increasing stress selection strategy was found not to be as resistant as spores exposed to the same increasing stress levels. Constant stress selection strategy also resulted in similar results where spores were more resistant than hyphae at the same stress level.

To summarize, mutant generations were obtained at two selective conditions for each morphological states. It was found that the fungus spores were found to be much more resistant to heat stress than hyphae. Mutant populations obtained from both selection strategies should be analyzed in detail to understand the mechanism of stress resistance in spore state and to reveal the presence of any stress-protectant metabolite.

YÖNLENDİRİLMİŞ EVRİM YÖNTEMİ İLE *FUSARIUM OXYSPORUM'*UN ENDÜSTRİYEL ÖZELİKLERİNİN GELİŞTİRİLMESİ

ÖZET

Bu çalışmada evrimsel mühendislik yöntemi kullanılarak *Fusarum oxysporum*'un ve bu organizmadan elde edilen lipazın sıcaklığa karşı direncinin artırılması amaçlanmıştır. Bu amaçla, yaban tip hücreler, bir kimyasal mutajen olan etil metan sulfonat (EMS)'ye maruz bırakılmıştır. Mutasyon ile elde edilen genetik çeşitliliği artırılmış hücrelere, sabit düzeyde ve dereceli olarak artan düzeylerde olmak üzere iki ayrı stres seleksiyon stratejisi uygulanmıştır. Her aşamada hayatta kalan bireylerin bir sonraki nesile aktarılması ile seçim stratejileri nesiller boyunca devam ettirilmiştir. Seçilim şartları küfün her iki morfolojik -hif ve spor- haline de ayrı ayrı uygulanmistir. Nesiller bu iki farkli morfolojik durumdaki küfe uygulanan stres sonrasında elde edilmişlerdir. Stres şartları kademeli olarak artan sıcaklık ile hafif siddette sabit sıcaklık uygulamaları olarak seçilmiştir. Son nesiller bu iki sıcaklık stresinin hem hif halindeki hem de spor halindeki küfe uygulmasiyla elde edilmişlerdir. Hif halindeki küfün artan sıcaklık stresine maruz bırakılan son neslinin, aynı küfün spor haline kıyasla aynı stres şartlarında daha az dirençli olduğu görülmüştür. Sabit olarak uygulanan sıcaklık stresinde ise yine hif halindeki küfün, spordan elde edilen aynı sıcaklık stresine maruz bırakılmış sporlardan daha az dirençli olduğu görülmüştür.

Özet olarak, küfün her iki morfolojik durumunda mutant nesiller elde edilmişlerdir. Spor halindeki küfün hif durumundakine göre sıcaklık stresine karşı daha dirençli olduğu ortaya çıkmıştır. Bu bilgiler ışığında, elde edilen mutant populasyonların spor halindeki stress direncinin mekanizmasının anlaşılabilmesi ve olası mevcut stresten koruyucu metabolitlerin belirlenmesi için detaylı bir şekilde analiz edilmesi gerekmektedir.

1. INTRODUCTION

1.1. Introduction to *Fusarium oxysporum*

Fusarium oxysporum is a common soil fungus which belongs to ascomycete group of filamentous fungi. The eukaryotic organism is found in all parts of the world as a pathogen to various different plant species as a coloniser of root surfaces of many plants and saprophytic growth on dead organic matter (Namiki et al, 1994). *F. oxysporum* causes economically important losses on a wide variety of crops and has also been reported as an opportunistic human pathogen (Delgado-Jarana et al, 2005). Systematic classification of *Fusarium oxysporum* is given in Table 1.1 according to NCBI Taxonomy.

Table 1.1: Systematic classification of *F. oxysporum*

Kingdom	Fungi
Phylum	*Ascomycota*
Class	*Sordariomycetes*
Order	*Hypocreales*
Family	*Nectriaceae*
Genus	*Fusarium*
Species	*F.oxysporum*

Plant pathogenic form of *Fusarium oxysporum* causes vascular wilt disease by invading plant's root from which fungus reaches xylem tissue, so it grows from roots to sap of

plant. Due to the fungal growth within plant's vascular tissue, water can not reach to the leaves. This lack of water induces the leaves' stomata to close, the leaves wilt, and the plant eventually dies (Agrios, 1988). There are over 80 known strains that show specific pathogenicity to particular crops, causing the vascular wilt diseases. Based on its host specificity, *F.oxysporum* isolates have been classified into *formae speciales* (Namiki et al, 1994). The strains that specifically affect banana are termed *F. oxysporum* f. sp. *cubense*, those that affect tomato are *F. oxysporum* f. sp. *lycopersici*, and those on peas are *F. oxysporum* f. sp. *pisi*, etc. They are morphologically indistinguishable thus the only way to understand the difference is pathogenicity test. Such pathogenic variation suggests an extreme genetic divergence within the species despite its highly conserved morphology (Namiki et al, 1994).

Ascomycetes have both sexual and asexual life cycles. However, genetic diversity of *F.oxysporum* has been explained by two hypothesis one of which claims that *F.oxysporum* reproduces asexually with mutations being the sole source of genetic diversity (Koenig et al. 1997, Bentley et al. 1998). The other claims that unidentified mechanisms exist that generate genetic variability; these mechanisms are attributed to the parasexual life cycle, form of mitotic recombination (Leslie 1993, Kuhn et al. 1995, Taylor 1999). Proving the hypothesis, hyphal fusion between two hyphae between two genomes was visualized by fluorescence microscopy (Wisser, 2000).

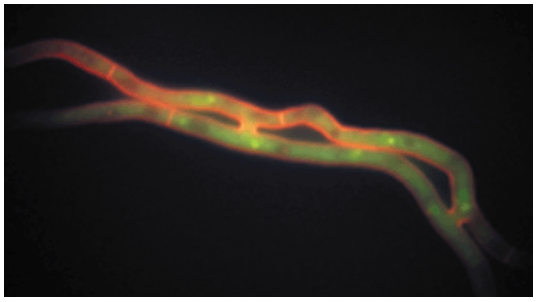

Figure 1.1: Hyphal fusion of *Fusarium oxysporum* f. sp. *cubense* (http://www.people.cornell.edu/pages/rjw29/foc.html)

F. oxysporum produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Agrios, 1988). Microconidia are one or two celled, and are the type of spore most abundantly and frequently produced by the fungus under all conditions. It is

also the type of spore most frequently produced within the vessels of infected plants. Microscopic view of *F.oxysporum* microconidia are shown in Figure 1.2.

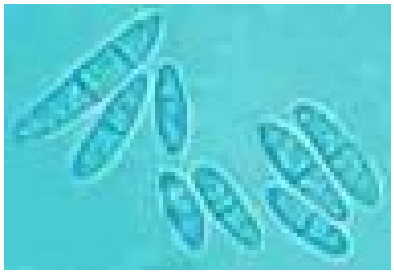

Figure 1.2: Microconidia of *Foxysporum* (http://www.rbgsyd.gov.au)

Macroconidia are three to five celled, gradually pointed and curved toward the ends. These spores are commonly found on the surface of plants killed by this pathogen as well as in sporodochialike groups. Microscopic view of *F.oxysporum* macroconidia is shown in Figure 1.3.

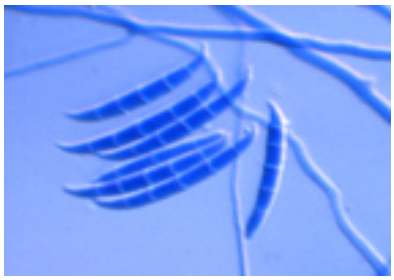

Figure 1.3: Macroconidia of *F.oxysporum* (http://www.med.univ-angers.fr)

Chlamydospores are round, thick-walled spores, produced either terminally or intercalary on older mycelium or in macroconidia. These spores are either one or two celled. Microscopic view of *F.oxysporum* chlamydospores are shown in Figure 1.4.

Figure 1.4: Chlamydospores of *F.oxysporum*
(http://www.inta.gov.ar/imyza/info/gal/galimage.htm)

In solid media culture, such as potato dextrose agar (PDA), the different special forms of *F. oxysporum* can have varying appearances. In general, the aerial mycelium first appears white, and then may change to a variety of colors - ranging from violet to dark purple - according to the strain (or special form) of *F. oxysporum* (Smith, 1988). Spore formation is easily observed on solid agar. However, sporulation is rarely achieved during submerged cultivation, due to relatively good nutrient availability and the partially physical nature of hyphal wall (Znidarsic et al., 2001).

F.oxysporum, as a common feature of filamentous fungi, grows in submerged cultures in the form of filaments, called hyphae. Fungal hyphae grow as it is polarized and grown out of a single cell. The tips are grown out of the main hyphae and produce branches which resulted in hyphal network, called mycelium. Hyphal tip extension requires several enzymes for both degradation of substrates which are absorbed through the fungal cell envelope (Baron, 1996) and synthesis of new cell wall. Hyphae formation starts with germination while hyphae tip acquires necessary proteolytic, glycolytic, lipolytic, synthetic enzymes besides cell wall subunits supplied in vesicles at the apex transported by cytoskeleton. Finally, the cell wall determines the shape of the cell (Znidarsic et al, 2001). Since the hyphal tip growth is closely related to protein secretion, the phase of growth and the state of enzyme production can be implied from the number of branches on hyphae.

From an industrial point of view, therefore, morphology of mycelium has a key role in the production of extracellular enzymes and recombinant proteins (Znidarsic et al, 2001).

1.2. Heat Response Mechanism of Fungi

Cells tolerate several different stresses by a common mechanism which is trehalose accumulation. Trehalose is a nonreducing disaccharide in which two glucose molecules are linked by α,α-1,1-glycosidic linkage which is shown in Figure 1.5.

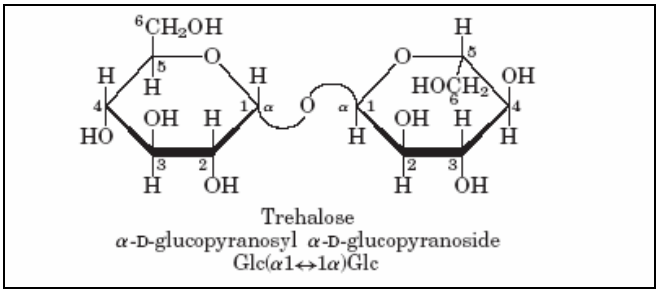

Figure 1.5: α,α-1,1-trehalose. (Lehninger, 4th Ed.)

This sugar is found in various organisms such as bacteria, yeast, fungi, insects, and invertebrates, higher and lower plants. Trehalose plays a dual role as an energy and carbon reserve mainly stored in vegetative resting cells and reproductive structures and as a stress metabolite. In addition to being nonreducing, it possesses several unique physical properties, which include high hydrophilicity and chemical stability, and the absence of internal hydrogen bond formation. These properties are the main cause of trehalose for being a stress metabolite (Argüelles, 2000; Plourde-Owobi et al., 2000)**.** In lower eukaryotes, trehalose content constitutes up to 15% of the dry weight of stationary phase cells or spores (Elbein, 1974). Trehalose accumulation also takes place in cells exposed to several stresses such as heat, ethanol and heavy metals. This metabolic activity is common to developing fungal spores and heat stressed cells. In *Aspergillus nidulans*, trehalose is important for hydrogen peroxide and thermo-tolerance (Fillinger et al., 2001).

In *S. cerevisiae*, trehalose is synthesized by the trehalose-6-phosphate synthase (TPS) complex and it is degraded by two trehalases, a cytosolic neutral and a vacuolar acid trehalase. The cycle of synthesis and degradation is important for the increased stress tolerance generated by trehalose, as mutation of the TPS genes as well as the trehalase genes causes a decrease in stress resistance (Smits, 2005)

Cellular effects of heat shock are mainly on respiration. Mitochondrial functions and structures are highly affected by heat shock. Physiological response of heat shock reduces respiratory ATP production and results in lag in protein import to mitochondria. However, it has been reported that this initial effect was diminished during prolonged

heat shock in *N. crassa.* Another effect at molecular level is the inhibition of precursor mRNA splicing which results in inhibition of mature mRNA production and protein synthesis (Jennings, 1993)

Molecular basis for adaptation of organisms to high temperature and other stresses have been analyzed and found that organisms exposed to the stress of high temperature synthesize a unique group of proteins. These heat shock-induced proteins have been shown to help cells adapt to inducing temperature and to survive exposure to higher, otherwise lethal temperatures. It has been discovered that many of the heat shock proteins are also synthesized by cells at normal temperature. There are four main groups of heat shock proteins synthesized in almost all organisms. Hsp70, hsp83/90, hsp100, α–crystallin-related small heat shock proteins in addition to hsp60 and ubiquitin. These proteins function in proper conformational folding of cellular proteins, assembling of protein complexes and translocation of protein complexes into target organelles by interacting with them. Due to the effect on conformation of other proteins, these heat shock proteins are called molecular chaperons. In order to understand the induction mechanism of heat shock gene transcription, heat shock transcription factors have been extensively studied to identify and characterize important factors, regulation conditions in different organisms (Jennings, 1993).

1.3. Industrial Importance of *Fusarium oxysporum*

F.oxysproum secretes extracellular enzymes into the environment in the process of biodegradation of plant cell wall. The organism has been utilized for its wide range of extracellular enzyme production especially for its lipolytic and glycolytic enzymes with improved characteristics in large amounts.

Enzymatic hydrolysis of lignocellulosic raw material and bioethanol production has been achieved since *F.oxysporum* has the ability to produce wide range of cellulases and xylanases. Direct conversion of biomass to ethanol makes *F.oxysporum* industrially important microorganism, in particular, in agricultural area (Panagiotou et al., 2005; Kuhad, 1998).

Lipases produced by special forms of *Fusarium oxysporum* could show different features in terms of selectivity. For instance; *F.oxysporum* f.sp.vasinfectum showed higher activity towards middle-chain triglycerides (Rapp, 1995). One of *F.oxysporum forma speciale* showed one of the highest lipase productions among 37 fungi so was recognized as good lipase producer. (Rapp, 1992) This specificity of lipase has been exploited in detergent, food and pharmaceutical industries replacing chemical reactions by biological ones.

Esterases with various catalytic features produced by *F.oxysporum* have also been studied for the benefit of industrial applications. For instance, acetyl esterase produced by *F.oxysporum* involved in xylan breakdown which is useful for different industrial processes, animal nutrition and carbohydrate synthesis was characterized and its production was optimized by P. Christakopoulos et al. (Christakopoulos et al., 1999).

Lipolytic enzymes produced by *F.oxysporum* have been extensively studied over the decade mostly for treatment of mechanical pulp slurries to reduce pitches formed during bleaching process of resins. It has been shown that the fungus is a good candidate for the degradation of wood sterol esters and resins (Tamerler and Keshavarz, 2000).

1.4. Lipases

Lipases are triacylglycerol acylhydrolases (E.C.3.1.1.3) which catalyze the hydrolysis of fats into fatty acids and glycerol at the water lipid interface and reversing the reaction in non-aqueous media. Catalytic action of lipase is shown in Fig 1.6.

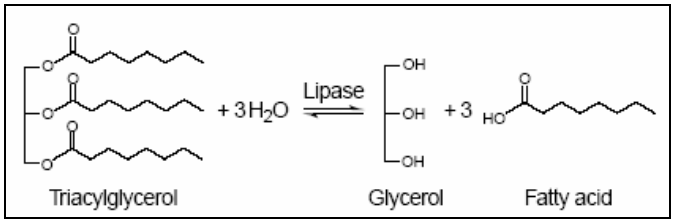

Figure 1.6: Catalytic action of lipase (Saxena et al., 1999)

There are two enzymes considered in breakdown of ester bonds, lipases and esterases. Although a clear distinction has not been established, lipases differ from esterases in two ways. One of which is their mode of action i.e. lipases hydrolyses water-insoluble

glycerides at water-oil interface whereas esterases act on water soluble esters in water (Fig 1.7) (Tamerler and Keshavarz, 2000; Rapp, 1992**).** The other is that as the majority of lipases exhibit high activities toward lipids with fatty acid residues of C_8-C_{18} chain length, esterases are highly active toward short-chain water soluble glycerol esters (Rapp, 1995).

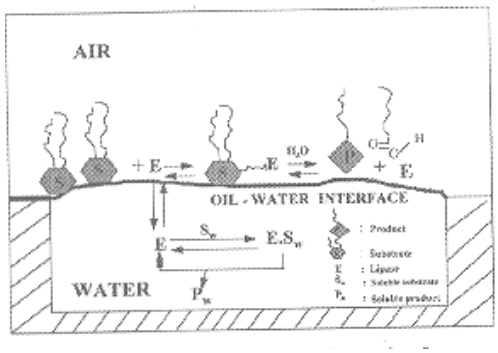

Figure 1.7: Lipolytic reaction at the oil–water interface. (Saxena et al., 1999)

In abundant presence of water in which enzyme is dissolved, lipase catalyses hydrolysis of ester bonds at the interface. On the other hand, in the absence of water under certain experimental conditions, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol (Saxena, et al., 1999). They catalyse a wide range of reactions, interesterification, alcoholysis, acidolysis, aminolysis (Pandey et al., 1999).

Studies on determining 3-D structure of lipase have been carried since the first lipase from *Rhizomucor miehei* and the human pancreas were determined in 1990 (Peters, 1997). All exhibit a characteristic folding pattern known as the α/β-hydrolase fold. The lipase core is composed of a central β sheet consisting of up to eight different β strands (β1–β8) connected by up to six α helices (Jaeger and Reetz, 1998). Hydrolysis action of lipase is carried out by a consensus sequence G-X-S-X-G where G=glycine, S=serine, X1=histidine and X2= glutamic or aspartic acid. Due to constitutive nucleophilic serine content, lipases are named serine hydrolases and do not require cofactor. This pentapeptide structure is highly conserved in most of the lipases having different origins and forms characteristic β–turn–α motif named the nucleophilic elbow. Due to its industrial importance, structure-function relationships have been studied extensively so

far. According to present literature, lipase starts hydrolysis of a substrate from its nucleophilic elbow. The oxygen atom on serine residue at the catalytic site attacks on the carbonyl carbon atom of the ester bond. This leads to the formation of a tetrahedral intermediate stabilized by hydrogen bonding to nitrogen atoms of main-chain residues that belong to the so called oxyanion hole. An alcohol is liberated, leaving behind an acyl–lipase complex, which is finally hydrolysed with liberation of the fatty acid and regeneration of the enzyme. Moreover, hydrolytic activity of the enzyme is greatly enhanced by the presence of insoluble substrates known as interfacial activation. During reaction, a change in conformation of the enzyme occurs. In other words, 3-D structures have shown that lipase possesses a lid-like structure (Fig 1.8.) which covers active site. Upon binding to interface, the lid moves away while a large hydrophobic surface is exposed which facilitates binding of lipase to interface, then active site becomes accessible to solvent (Jaeger and Reetz, 1998). According to more recent researches, nevertheless, it has been shown that the presence of amphiphilic lid is not necessarily related to interfacial activation. Lipase can be active without water-oil interface (Carmen Ban'o, 2003).

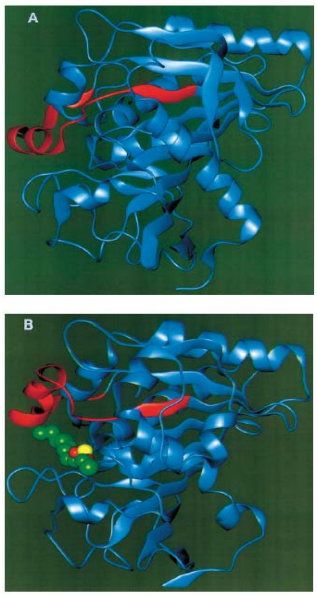

Figure 1.8: Secondary structures of (a) inactive and (b) active (inhibitor- bound) conformations of the *R.miehei* lipase.

The red region indicates the lid region (Fig.1.8). In the active form (B)ethyl-decanoylhosphate is covalently bound to the active serine (Ser144). Green, red and yellow represent the methyl(ene),oxygen and phosphorus atoms respectively (Peters et al, 1997).

The Lipase Engineering Database (LED; http://www.led.uni-stuttgart.de) provides information on sequence- structure-function relationships of various microbial lipases.

Lipases are produced by animals, plants, filamentous fungi, yeast and bacteria. In fact, over hundred years, microbial lipases have taken great deal of attention (Rapp, 1992). Due to their thermotolerance, stability, selectivity and broad substrate specificity providing utilization of many unnatural acids, alcohols or amines as substrates, they have a great potential for commercial applications (Cardenas et al., 2001). Besides their multifold properties, microbial lipases are preferred also for their easy extraction procedures and unlimited supply. As a result, there is impressive number of reviews covering microbial lipases having different catalytic properties from various origins. Each year around 1000 original articles are published on lipase. This shows the interest in lipase among biocatalysts (Jaeger and Eggert 2002; Bornscheuer 2002; Saxena 1999; Jaeger and Reetz, 1998).

Extensive studies for searching lipases having novel characteristics have been carried out over years in consequence of increasing industrial interest. Major screening studies to find high lipase producing microorganisms were carried out and published (Rapp et al., 1992; Garcia-Lepe et al., 1997; Cardenas et al., 2001).

Among microbial species, fungi are preferable lipase sources because fungal enzymes are usually secreted extracellularly which makes bioseparation process easier and cheaper. There are several comprehensive reviews published covering fungal lipases studied since 1950s**.** Fungal lipases are preferred not only because of their easy extraction but also for their thermal and pH stability, substrate specificity, activity in organic solvents. Therefore, they have been extensively studied both from the biochemical and the genetic point of view. The most productive species belonged to the genera are found to be *Geotrichium, Penicillium, Aspergillus, Rhizomucor, Candida, Humicola* (Cardenas et al., 2001).

Lipase production by different *Fusarium* species has been well documented (Rapp et al, 1992). In particular, the production of lipase by several *Fusarium* strains has been studied in terms of enzyme production, protein properties and purification (Maia et al., 1999; Nagao et al., 2000). *Fusarium oxysporum f.s vasinfectum* has been shown as one of the best lipase producer. *F.oxysporum f.sp.lini* known to produce unique intracellular and extracellular lipases was stimulated by infusion of intracellular Ca^{+2} ions resulting in extracellular lipase release (Hoshino et al., 1991).

Microbial diversity is providing numerous possibilities to obtain lipase with different properties. Over fifty years, best microbial lipase producers have been screened and characterized. Moreover, physiological conditions to achieve the best activity during production of lipase have still been investigated. In present day, microbiologists are still searching for new lipase producing species at extreme environments (Haki, 2003). Along with the search in nature, the enzyme is engineered for the desired properties based on crystallographic and catalytic knowledge.

1.4.1. Temperature and pH Optima

Lipase optimum working pH changes between 7.0-9.0 depending on its source. However, there are some exceptional enzymes produced by *Aspergillus niger*, *Chromobacterium viscosum* and *Rhizopus* sp. whose lipases are active at low pH. High stability is found to be around pH 6.0–7.5. Moreover, alkaline lipases are also available in nature produced by organisms living in basic environments such as *P. nitroreducens* whose lipase is active at pH 11.0 (Saxena et al, 1999).

Most lipases show maximum activity temperatures of 30-40°C (Bornscheuer et al, 2002; Saxena et al., 2002). Lipases from thermophilic organisms in nature tend to be stable at higher temperatures. Thermophilic bacterial lipases from the genera Bacillus and those isolated from from Icelandic hot spring showed higher lipase activity at 40°C to 60°C (Sharma et al., 2002; Saxena et al., 2002). To give an idea , while lipases of *A. niger*, *R. japonicus*, and *C. viscosum* are stable at 50°C, lipases of thermotolerant *H. lanuginosa* and *P*. sp. *nitroreducens* are stable at 60°C and 70°C , respectively.

1.4.2. Industrial Importance of Lipase and Commercialized Products

1.4.2.1. Food Industry

Industrial enzymes, especially lipases, are commonly used in the production of a variety of products, ranging from fruit juices, baked foods and vegetable fermentation to dairy enrichment. Microbial lipases have been used for the production of desirable flavours in cheese and other foods, and for the interesterification of fats and oils to produce modified acylglycerols, which cannot be obtained by conventional chemical interesterification (Pandey et al., 1999). In the production of artificial cocoa butter, lipase replaces palmitic acid with stearic acid through transesterification ending up with stearic-oleic-stearic acid as a cocoa butter (Schmidt et al, 1999).

Current applications include flavour enhancement of cheese, acceleration of cheese ripening, manufacture of cheese-like products, and lipolysis of butter fat, and cream. Cheese made from ewe's or goat's milk is imitated by addition of lipases to cow's milk so-called enzyme-modified cheese (EMC) used as additive to sauces, dips, ready soups

(Saxena et al., 1999). Lipases are also useful in the synthesis of the artificial sweetener sucralose by regioselective hydrolysis of octa-acetylsucrose.

1.4.2.2. Detergent Industry

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. Lipases are used mostly in combination with other enzymes. The world-wide trend towards lower laundering temperatures has led to much higher demand for household detergent formulations. Studies continue to meet the needs of detergent industry in terms of improving working pH, temperature, stability in organic solvents and substrate range of lipase through combination of continuous screening for improved lipases and protein engineering. Since 1994 along with scientific researches, lipases which have been developed by recombinant DNA or directed evolution techniques have been commercialized by several companies. The first lipase was introduced by Novo Nordisk to the market. The enzyme was originated from the fungus *T. lanuginosus* and was expressed in *Aspergillus oryzae*. Lumafast™ from *Pseudomonas mendocina* and Lipomax™ from *Pseudomonas alcaligenes*, both produced by Genencor International are the following commercial enzymes (Jaeger and Reetz, 1998).

Unilever, Cosmo Oil and Procter & Gamble, Biocatalyst, Amano are major companies working on recombinant DNA technology for surfactant lipases. Thus, lipase mediated detergents are promising with the use of recombinant technology and have an increasing market share in detergent industry (Pandey et al., 1999).

1.4.2.3. Paper and Pulp industry

The hydrophobic components of wood, namely triglycerides and waxes, cause severe problems in pulp and paper manufacture. Lipase treatment was introduced to replace chemical degradation. As the first commercial product, Nippon Paper Industries in Japan developed a pitch control method that uses a fungal lipase from *C. rugosa* to hydrolyse

up to 90% of the triglycerides commercially known as Resinase [R,] Novozyme. Currently, there are quite a number of commercial products especially developed for this industry.

Table 1.2: Commercial enzyme preparations (Kontkanen et al., 2004)

Lipase preparations used in the present work

Origin	Supplier	Market name
Aspergillus niger	Amano	Lipase A "Amano"6
Aspergillus niger	Biocatalysts	L018
Aspergillus sp.	Novozymes	Resinase A
Candida antarctica	Novozymes	Novozym 525L
Candida rugosa	Amano	Lipase AY "Amano"30
Candida rugosa	Biocatalysts	L034
Chromobacterium viscosum	Asahi	Lipase (LP)
Geotrichum candidum	Biocatalysts	L052
Mucor javanicus	Amano	Lipase M "Amano"10
Penicillium camembertii	Amano	Lipase G "Amano"50
Penicillium cyclopium	Biocatalysts	L055
Penicillium roqueforti	Amano	Lipase R "Amano"
Pseudomonas sp.	Amano	PS
Pseudomonas sp.	Seppim	Lipase PS-30
Rhizopus arrhizus	Biocatalysts	L057
Rhizomucor miehei	Novozymes	Palatase 20000L
Rhizopus niveus	Amano	Newlase F
Rhizopus oryzae	Amano	Lipase F-AP 15
Thermomyces lanugenousus	Novozymes	Lipozyme TI 100L

1.4.2.4. Cosmetic and perfume industry

Flavor, aroma and surfactant productions are the main processes in cosmetics and perfume industry. Monoacylglycerols and diacylglycerols are useful as surfactants in cosmetics. These compounds are prepared by the lipase-catalysed esterification of glycerol. In addition, transesterification of alcohol esters with lipase from various microbial sources was achieved to prepare rose oxide which is an important fragrance ingredient in perfume industry (Pandey et al, 1999). Another important perfumery constituent, the plant growth factor (–)-methyl jasmonate, is synthesised with a lipase catalysed reaction using the commercially available Lipase P produced by Amano Co. to yield the chiral key intermediate (Jaeger and Eggert, 2002).

Since they give soothing to the skin in personal care products like sun-tan creams, bath oils, isopropyl myristate, isopropyl palmitate, and 2-ethylhexyl palmitate were started to be produced by Unichem International using lipase. Wax esters have similar application in personal care products and are being manufactured enzymatically (Saxena et al.,

1999). Two patents are available belonging to Asahi-Electrochem for the production of maltose-like and lactose-like sugar fatty acid esters. These esters have numerous applications, for example in cosmetics and medicines. The production of glycerine mixtures, used as an external preparation or by application directly to skin was achieved by lipase utilization and the enzyme was patented (Pandey et al., 1999).

1.4.2.5. Pharmaceutical industry

Conventional chemical synthesis of drugs containing a chiral centre generally yields equal mixtures of enantiomers. During the past decade, many studies have shown that racemic drugs usually have the desired therapeutic activity residing mainly in one of the enantiomers and the other enantiomers might interact with different receptor sites, which can cause unwanted side effects.

Production of chiral drugs including amoxicillin (an antibiotic), captopril (an angiotensin-converting-enzyme inhibitor) and erythropoietin (the haematopoietic growth factor) is, becoming more and more important (Jaeger, 1998). Resolution of racemic mixtures is carried out by biocatalysts especially by lipase owing to its high stereoselectivity. Some common examples are as follows; resolution of racemic alcohols in the preparation of some prostaglandins, steroids and carbocyclic nucleoside analogues, pharmacologically active S-enantiomer selection of profens (2-aryl propinoic acids) that is an important group of nonsteroidal anti-inflammatory drugs, R-isomer of ketoprofen in an achiral solvent such as isobutyl methyl ketone and (S+)-carvone (Rohit et al, 2001). Regioselective modification of polyfunctional organic compounds is yet another rapidly expanding area of lipase application, particularly in the field of AIDS treatment. Large scale vitamin production has been mediated by regioselective lipase-catalysed systems by Roche Vitamins. Consequently, considerable efforts are being made to obtain optically pure compounds in pharmaceutical industry.

1.4.2.6. New Biopolymeric Material Production

Biopolymers like polyphenols, polysaccharides and polyesters show a considerable degree of diversity and complexity. Furthermore, these compounds are receiving increasing attention because they are biodegradable and produced from renewable

natural resources. Lipase is used as catalysts for polymeric synthesis (Jaeger and Eggert, 2002).

1.4.2.7. Biodiesel Production

An alternative source of energy for public transport is the so-called biodiesel, which has been produced chemically using oil from various plants (e.g. rapeseed). Biodiesel fuel originates from renewable natural resources and concomitantly reduces sulfur oxide production. The conversion of vegetable oil to methyl- or other short-chain alcohol esters can be catalysed in a single transesterification reaction using lipases in organic solvents. However, the production at an industrial scale failed so far because of the high cost of the appropriate biocatalyst (Jaeger and Eggert, 2002). One of the successful applications of lipase for biodiesel production was achieved by Shimada et al. They used vegetable oils and immobilized *Candida antarctica* lipase to derive biodiesel (Shimada et al., 1999).

1.4.2.8 The Need for Thermostable Lipases

Most of the industrial processes in which lipases are employed function at temperatures exceeding 45^0C. One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Allowing a higher operation temperature has also a significant influence on the bioavailability and solubility of organic compounds and thereby provides efficient bioremediation (Becker, 2003). Other values of elevated process temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and higher process yield due to increased solubility of substrates and products and favorable equilibrium displacement in endothermic reactions. The enzymes, thus, are required to exhibit an optimum temperature of around 50^0C (Sharma et al., 2002).However, there are some extarordinary processes that take place above 50^0C such as seed oil refinement process taking place at 65^0C. Although, few lipases exist which are able to operate at 100^0C, their half-lives are reported to be short (Rathi et al., 2000).

The industrial demand for lipases well active at high temperature continues to stimulate the search for thermostable enzymes producers among microorganisms. On the other hand, engineering and evolution studies continue to manipulate existing sources of DNA fragments to enhance thermostability and activity at elevated temperatures. Thermophilic enzymes can also be used as models for the understanding of thermostability and thermo-activity for protein engineering studies. Rational approaches are conducted to understand thermophilic enzyme structure by comparing structure and homologues genes with mesophilic lipases. Hence, in order to meet the need for more stable lipase produced in large quantities, a lot of efforts are being made in nature and also in laboratories.

Table 1.3: Industrial use of microbial lipases (Vulfson, 1994)

Industry	Action	Product or Application
Detergents	Hydrolysis of Fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents, milk, cheese and butter
Bakery foods	Flavor improvement	Shelf life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality Improvement	Mayonnaise, dressings and whippings
Food dressings	Quality Improvement	Mayonnaise, dressings and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor improvement	Meat and fish products fat removal
Fats and oils	Transesterification, Hydrolysis	Cocoa butter, margarine fatty acids, glycerol, mono and diglycerides
Chemicals	Enantioselectivity, Synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Speciality lipids, digestive aids
Cosmetic	Synthesis	Emulsifiers, Moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of Fats

1.5. Rational Metabolic Engineering

Metabolic engineering is defined as the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology (Stephanopoulos, 1988)

Main objective of metabolic engineering is to understand metabolic pathways through the information of biochemical reactions. For this purpose, metabolic engineering contributes to metabolic flux analysis by using chemical engineering concepts and flux control using molecular biology techniques to conventional metabolic map studies. Since it has been only little revealed about metabolic pathways through mapping only by tracing carbon, nitrogen and energy fluxes under a particular set of conditions, metabolic engineering concept was based on *in vivo* analysis of metabolic flux under specific conditions, in other words, the degree of a pathway in the whole metabolic network. Metabolic engineering is focused on the whole system instead of its constituent parts (Stephanopoulos, 1988).

Metabolic flux analysis is a major component of metabolic engineering. This approach, developed in 1970's, is extended to analysis of metabolic reactions around branch points in metabolic networks instead of analyzing every single reaction step. Metabolic flux *in vivo* analysis involves several techniques and methods. These approaches are based on material balances, the measurement of label enrichment in selected secreted metabolites, analysis of the fine structure of NMR spectra, and measurement of isotopomer molecular weight distributions by gas chromatography-mass spectrometry (Stephanopoulos, 1999).

There are many applications of metabolic engineering involving the enhancement of product yield, xenobiotic degradation, and improvement of cellular properties, extension of product spectrum and novel product invention and substrate range extension, analysis of the metabolism of whole organs and tissues as well as the identification of targets for disease control by gene therapy (Stephanopoulos, 1999; Wang et al., 2000).

Metabolic engineering is based on the knowledge of metabolic system of interest including reaction kinetics, intermediate metabolite concentrations or well-designed

stimulus-response experiments. In some cases, even though the genetic experiments are well-established, the final phenotype differs from that desired through constructive metabolic engineering (Bailey, 1991). Despite the fact that metabolic engineering has a wide application area in biotechnology and provides numerous genetic possibilities that are attainable, the possible gene manipulations are effective only in small numbers. The main problems are identifying flux-limiting step in a specified metabolic pathway and the catalysts involved in.

1.5.1. Rational Metabolic Engineering Approach Toward Lipase Production

The production of lipases is increasingly achieved by expression in recombinant organisms, and a considerable number of lipase genes have been cloned and functionally expressed based on the knowledge of gene structure, computer design. The use of suitable expression systems, which often enable secretion of mature lipases into the cultivation medium, has made the effective production of large amounts of active lipase possible (Bornscheuer et al., 2002).

Many microbial lipase genes belonging to *Candida rugosa*, *Candida antarctica, Thermomyces lanuginosa (formerly Humicola), Rhizomucor miehei, Rhizopus delemar, Geotrichum candidum, Burkholderia cepacia (formerly Pseudomonas), Pseudomonas pseudoalcaligenes, Pseudomonas mendocina (originally P. putida),* C*hromobacterium glumae* (identical to the lipase from Burkholderia glumae), including important commercial lipases like *(formerly C. cylindracea),* were cloned during the past years (Schmidt-Dannert, 1999).

Overexpression of cloned lipase genes in suitable hosts makes the production of most appropriate lipases for specific applications available facilitating easy purification of enzyme from other proteins in the culture. Bacterial lipases from various *Bacillus* species can be overexpressed in *E. coli* using conventional overexpression systems. Some filamentous fungi and yeasts are also chosen as good hosts for heterologous expression of lipase coming from another origin. Lipase from *Rhizomucor miehei* can be produced in the fungi *Aspergillus oryzae* (Bornscheuer, 2002). Other than *A.oryzae, S.cerevisae, P. pastoris* are widely used host organisms as well as *E.coli* (Schmidt-

Dannert, 1999). One of major drawbacks is that folding and secretion of lipase are highly specific processes that normally do not function properly in heterologous hosts, as in the case of lipase expression from *Pseudomonas* species which requires the functional assistance of about 30 different cellular proteins before they can be recovered from the culture supernatant in an enzymatically active state (Jaeger and Eggert, 2002).

In an impressive example, site-directed mutagenesis was successfully applied on *C.antarctica* B-lipase gene resulting in twofold improvement of the enantioselectivity (E) of the *C. antarctica* B-lipase catalyzed resolution of 1-chloro-2-octanol by the exchange of a single amino acid (Ser47Ala), as predicted by molecular modeling (Rotticci et al., 2001).

According to another study based on structure-function relationship of lipase, *F.heterosporum* lipase cDNA was expressed in *S.cerevisia,* resulting in improvement of thermal stability. Two fractions A and B belonging to lipase were obtained through expression of lipase gene in another host without being modified after translation. Therefore, lipase B that is thermally more stable and not produced in supernatant by original strain due to post translational modification was obtained. In addition, mutant lipases were produced by applying certain deletions and amino acid replacements in the related gene where protease cleavage site locates (Nagao et al., 2002).

In brief, recombinant studies to improve the specific catalytic properties of lipase are limited by few species and expression systems. Yet, there are good examples of achievements in recombinant production such as overexpression of *R.oryzae* having industrially important catalytic 1,3-regio-specificity, improved substrate specifcity of *G.candidum* lipase B and improvement in the stability as well as the activity at elevated temperatures of *B.thermocatenulatus* lipase (Schmidt-Dannert, 1999).

Despite the fact that recombinant lipase production facilitates better isolation of lipase isoforms secreted varying amounts or under different cultivation conditions with various catalytic specificities, this technique requires not only detailed knowledge for lipase binding site, i.e structural information, but also design concepts to improve specific property such as substrate specificity or thermostability. Depending on these

requirements, rational engineering approaches have been only marginally successful in improving the lipase stability (Zhang et al., 2003).

1.6. Inverse Metabolic Engineering

A novel approach was introduced in 1990's to produce desired phenotypes without secondary effects on cellular behavior as a result of genetic modifications in individual pathways (Bailey, 1996). Based on gaining desired phenotype in the first place, the approach then continues with determining of the genetic basis of obtained phenotype and re-engineering it to industrial host. This alternative strategy is named as *inverse metabolic engineering* which is explained in detail in the next part.

1.6.1. Directed evolution approach

Directed evolution was proposed in mid-1990s as an alternative molecular biology approach to extend the possible industrial features of lipase. This concept that does not require any knowledge of the structure or the mechanism of an enzyme is also known *in vitro* evolution of enzymes.

The early example of directed evolution was conducted by Moore and Arnold on the hydrolysis of a para-nitrobenzyl ester of an antibiotic (Moore and Arnold, 1996). In 1994, the lipase from *T. lanuginose* expressed in *A.oryzae*, became the first commercial engineered enzyme. Novo Nordisk Lipolase was engineered through directed evolution to meet the needs of detergent industry in terms of wash performance in detergents (Schmidt-Dannert ,1999 ; Jaeger and Reetz, 1998).

Directed enzyme evolution generally begins with the creation of a library of mutated genes. Gene products that show improvement with respect to the desired property' or set of properties are identified by selection or screening, and the gene(s) encoding those enzymes are subjected to further cycles of mutation and screening in order to accumulate beneficial mutations. This evolution can involve few or many generations, depending on how far one wishes to progress and the effects of mutations observed in each generation (Kuchner and Arnold, 1997).

One of the key steps is creating a mutant library throughout the procedure. Mutations are accumulated in successive generations sequentially, or by sequential mutation coupled with random recombination which has been argued to be the most efficient means of evolution because it effectively avoids the accumulation of deleterious mutations through recombination between mutant and wild-type sequences (Cherry et al, 1999).

Alternative to multiple cycles of mutagenesis including UV mutation, chemical mutagenesis and error prone PCR following recombination, homologous recombination of DNA in vitro, was developed by Stemmer in 1994. The method involves digesting a large gene with DNase to a pool of random DNA fragments. These fragments can be reassembled into a full-length gene by repeated cycles of annealing in the presence of DNA polymerase. The fragments prime each other based on homology, and recombination occurs when fragments from one copy of a gene prime on another copy, causing a template switch (Stemmer, 1994; Kuchner and Arnold, I997). Presently, combination of error-prone PCR and DNA shuffling, are seen as outstanding methods (Reetz , 2004).

Secondly, the other key step is screening and selection of desired mutants. Identification is performed either by selection i.e. by a growth-related assay or by screening using high-throughput technologies. Despite the efforts to develop high-tech screening technologies, the availability of suitable systems for the identification of desired mutants out of a huge library containing like 10^3-10^8 variants is a major challenge. After the first application on the selection of mutant lipase catalyzing the enantioselective hydrolysis of the chiral ester from *Pseudomonas aeruginosa* (Reetz, 2004), microscale screening conducted in microtiter plates has been a solution which allows the rapid identification of active and stereoselective biocatalysts in a library in the past few years (Bornscheuer et al, 2002).

Among biocatalysts, lipase has been particularly an attractive target for directed evolution studies. Lipase was achieved to evolve successfully for higher stability, for higher enantioselectivity and for altered substrate specificity (Fujii et al, 2003). Since enantiomerically pure compounds are of rapidly increasing importance to the chemical industry as pharmaceuticals, agrochemicals, flavors and fragrances, lipase's

enantioselectivity property has been the focus of many studies. Fig 1.10 shows the algorithm of directed evolution for enantioselective lipase generation (Reetz et al, 2004; Bocola et al. 2004,; Jaeger and Reetz, 1998; Jaeger, 1999; Liebeton et al, 2000).

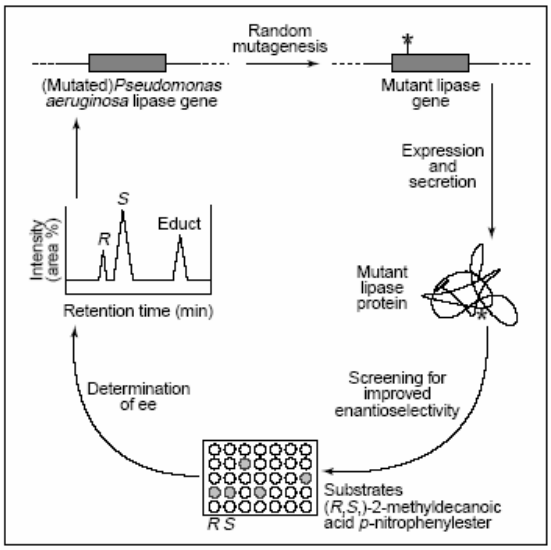

Figure.1.9: Strategy to create an enantioselective lipase by directed evolution (Jaeger and Reetz, 1998)

Novel approaches towards improvement of enantioselectivity are also available. Recently, it has been reported that the production of enantiomerically enriched compounds were separated by developing of a novel whole cell biocatalyst system containing surface display of lipase on *E.coli* cells (Lee et al*.,* 2004).

Thermostability enhancement studies have also been conducted by several groups. P*seudomonas aeruginosa* lipase was randomly mutated to obtain a thermostable lipase, but the stability increased by only 3^0C compared with the wild-type enzyme (Kuchner and Arnold., 1997)**.** Furthermore, in a study, it was achieved to generate *C.antarctica* mutants with more than 20-fold improvement in half-life at 70°C by applying error-prone PCR (Zhang et al., 2003**).** In a recent research, thermostability of *C.antarctica* lipase B, one of the most used biocatalysis in laboratory scale and commercial scale, was improved by 6.4^0C and its chimeric feature towards hydrolysis of DDG through DNA family shuffling.

Modern methods of genetic engineering combined with an increasing knowledge of structure and function allows further adaptation to industrial needs and exploration of novel applications. Recombinant DNA technologies allow the engineering of lipases for specific applications by altering their enantioselectivity, substrate specificity or general process performance. Rational design of lipases has been addressed mainly to the improvement of catalyst performance in laundry detergents. As a novel approach, directed evolution has been proved to be a rapid and yet powerful method to alter enzyme properties.

1.6.2. IME approach: Evolutionary Engineering

Evolutionary concept was first used for protein engineering studies as an alternative to rational design through defined, site-directed mutagenesis. The problems encountered in rational engineering approach have been overcome by evolution. Directed evolution concept has been introduced to improve protein functions. Repeated cycles of creation genetic diversity, examining the variants by screening or selection toward a desired phenotype constitute directed enzyme evolution. The achievements in directed enzyme evolution have lead to the use of this concept for entire pathways (Bailey et al., 1996; Sauer 2001).

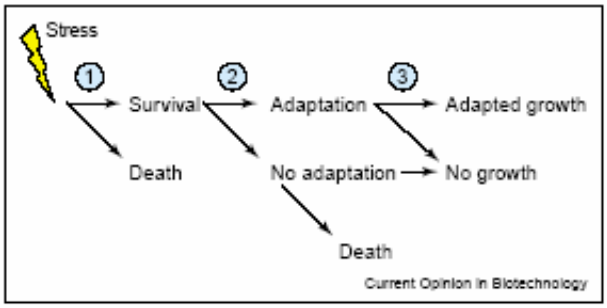

Figure.1.10: Schematic representation of the effects on survival (Smits and Brul, 2005)

Figure 1.10 indicates 1) When cells encounter a stressful situation (symbolized by lightning).Survival depends on initial resistance which is determined by genetic make-up and growth history of cell.2) If the cell survives it ill alter its physiology and gene expression program to adapt. It will need to both repair damage and to adapt to decrease

the effects of the continued stress. Whether or not this is successful depends on the genetic possibilities for adaptation and the energy that is available or that can be liberated.3) After successful repair and adaptation, the cell will grown again if enough resources are still available. Energetic requirements are increased for permanently stress-adapted growth (Smits and Brul, 2005).

The manipulation of several differing genes can result in the same phenotype because of the multi-factorial nature of many desirable phenotypes. Accordingly, *inverse metabolic engineering* is established to unravel complex phenotypes that are important in most bioprocess applications. The terminology was introduced by James Bailey (1996) to describe the approach from identifying phenotype to gene mapping while distinguishing it from *reverse genetics* used for identification of the gene which encodes a particular protein of interest. Inverse metabolic engineering strategy consists of three main steps. The first step is identifying, constructing or calculating a desired phenotype, second step is deciphering genetic basis of the new phenotype and finally engineering concept involves in it by transferring genetic basis in an industrial host organism (Bailey et al., 1996). Hitherto, some studies through inverse metabolic engineering have been conducted to improve pentose metabolism, (Becker and Boles, 2003), acetate resistance (Steiner and Sauer, 2003) phosphogen kinase systems (Sauer and Schlattner, 2004) and incorporation of unnatural amino acids (Bacher and Ellington, 2003).

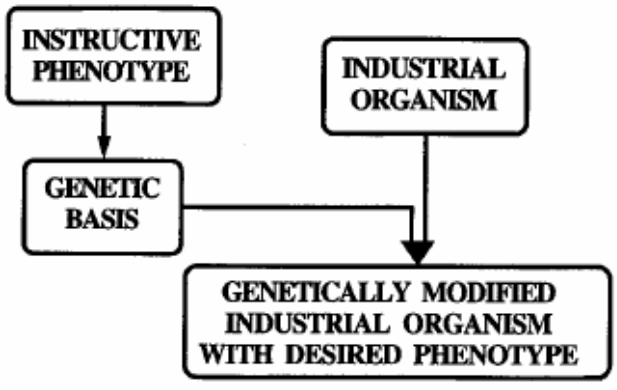

Figure 1.11: Schematic diagram of information flow in inverse metabolic engineering (Sauer, 2001)

In the light of established algorithm for inverse metabolic engineering, evolutionary engineering has extended the inverse metabolic engineering concept to recombination and continuous evolution of large populations over many generations from basic strain development by random mutation and direct selection on plates (Sauer, 2001). Recombination and continuous selection in large populations for many generations provide solutions to the problems prevailed by so called step-wise directed evolution of whole cells. Those obstacles are the accumulation of unfavorable mutations which eventually leads to crippled strains and the difficulty of selection by simply increasing resistance towards challenging agent or a condition such as resistance to toxic analogs of metabolic intermediates. By means of recombination, beneficial mutations from different variants could gather in one strain and decrease the size of deleterious mutation accumulation.

Continuous selection strategy increases probability of *in vivo* evolution of large populations. Genes and even entire operons are transferable as mobile genetic entities by intracellular or intercellular horizontal gene transfer. As soon as these genes or operons are acquired by an organism, recombination with related genes from other organism will be inevitable. Therefore, in large populations the degree of genetic variability is high because of increased possibility for genes transfer. Continuous selection of mixed populations containing genetically varied individuals under artificially posed conditions results in evolved population exhibiting desired phenotype (Sauer, 2001)

1.6.2.1. Genetic diversity

Organisms show differing mutability properties. For example, viruses are more susceptible to spontaneous mutations than microbial populations. However, there might be some exceptions like pathogenic organisms possessing hyper-mutable genes. In this way, *F.oxysporum* as a plant pathogen is considered to show high mutability through various specific mechanisms which is a functional property for evolutionary engineering strategy. Genetic diversity in a population is created by several means. Spontaneous mutations through several mechanisms, chemical and radiation-induced mutations, tagged mutagenesis are some of the ways used in literature for strain improvement. Horizontal DNA transfer, DNA rearrangements, small local changes are three main

mechanisms which cause spontaneous alterations. Environmental conditions can accelerate the rate of spontaneous mutations which are normally low and rather stable. Moreover, radiation and chemicals are widely used treatments for creating diversity for almost all organisms. UV and many chemical mutagens introduce the exchange of nucleotides or frame-shifts. Mostly used chemical mutagens are ethyl methane sulphonate (EMS), nitroso-methyl guanidine (NTG).The main reason to prefer UV, EMS and NTG in most applications is that they induce a great variety of molecular alterations with no apparent specificity for genomic subregions. In evolutionary engineering process, the rate of mutagenesis has to be appropriately arranged for an efficient diversified population since mutagenesis may have either no apparent or totally harmful effect. Optimum dose of mutagen is critical to obtain highest proportion of desirable mutants.

EMS is an alkylating agent that preferably alters G-rich regions in the genome. Mutagen causes depurination by modifying G residues. Alkylated bases can mispair during replication (Lawrence, 1991). Thus, modified G nucleotides can pair with T instead of C so G-C pairs turn into G-T pairs. Mismatched G-T pairs in the next replication cycle become A-T with the help of induced error-prone repairing system. Therefore, G-C pairs are replaced by A-T pairs. Codons for four amino acids: phe (UUY), tyr (UAY), asp (AAY), and lys (AAR) are mutable by EMS to other residues only at extremely low frequencies and codons such as those for proline (CCX) and glycine (GGX) are not normally produced by EMS mutagenesis (De Stasio, 2001).

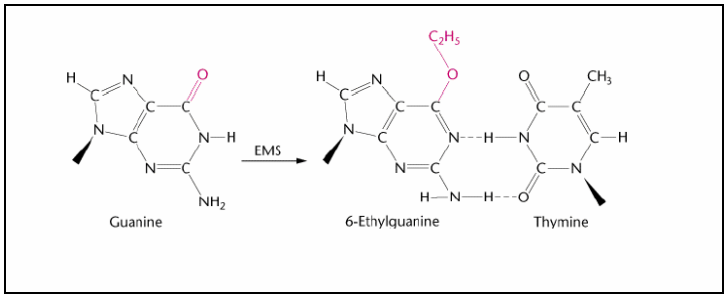

Figure 1. 12: Action of EMS on DNA
(http://www.mun.ca/biology/scarr/EMS_Mutagenesis.htm)

1.6.2.2. Selection

Selection procedure is the key step in inverse metabolic and evolutionary engineering strategies. Basically, phenotypes that show highest relative fitness in the population are screened and selected by using appropriate assays. Recently, technical advances have also provided high-throughput screenings with multi-well screening equipped with digital camera, robotic handling systems, and parallel photocells. In spite of easiness and rapidity, this step-wise evolution process has some drawbacks at some points; especially, unknown behaviors of identified phenotype under production process conditions. Continuous evolution brings a solution to this problem by applying selection procedure under process-relevant conditions. Selection in an environment having similar industrial process conditions, unexpected behaviors could be prevented (Sauer, 2001).

In order to improve extracellular enzyme production, chemostat selection has been used mostly. A successful example of this application was the selection of efficient protease secreting mutants by using bovine serum albumin as the sole nitrogen source based on microcolonies. Moreover, there are some achievements improving thermostable enzyme production simply by applying evolutionary engineering strategy. However, these procedures involved recombinant DNA technology. Thermostable enzymes expressed in thermophilic organisms and transformants were selected according to their activity-dependent survival at high temperatures (Sauer, 2001).

Short generation time of microorganisms makes evolutionary adaptation studies available so that natural evolution can be carried out in simple laboratory environment observing many generations. In a population, random mutagenesis creates diversity. Among various individuals, the fittest survives and reproduces. In other words, propagate its genetic material to future generations. Evolutionary adaptation studies are conducted mostly with *S. cerevisiae* which has been exploited for ages for alcohol production and baking. Recently, *S. cerevisiae* was evolved to resist multiple-stress including oxidative, freezing–thawing, high-temperature and ethanol stress (Çakar et al., 2005). Apart from improving already existing properties, *S. cerevisiae* was evolved to utilize xylose in an anaerobic environment as a novel gained function (Sonderegger and Sauer, 2003). There are some other studies with bacteria associating designing strains

with new products as well as enhanced resistance to environmental conditions. *Acetobacter aceti* was grown in long-term continuous culture to develop acetate resistance (Steiner and Sauer, 2003). Fungi are less exploited organisms for evolutionary studies. The species, especially filamentous fungi, have important morphological properties in fermentation technology. Mycelial growth is not a desirable characteristic, thus continuous growth and selection procedure was applied to *F. graminearum* simply by eliminating highly branched mutants in a low pH environment (Wiebe, 1996). Another important biotechnological aspect is thermal resistance and stability of whole cells and secreted enzymes. Despite of the fact that there are many studies to increase thermal resistance of extracellular enzymes, the strategies require large amount of information: i.e information on genetic sequences, translational modifications and functions of genes in metabolic pathways. At this point, evolutionary engineering strategy may be an alternative method providing much more stability, safety and public acceptance (Çakar et al., 2005).

Evolutionary studies on fungi are very rare in literature in comparison to yeast and bacteria. This approach could be much more effective to improve a desired phenotypic property of a fungus considering available insufficient genetic knowledge on fungi.

1.6.2.3. Genetic Analysis

In evolutionary engineering strategy, determining the genetic and metabolic differences between wild type and evolved strains is essential. Important determinants of the feasibility for elucidating the genetic basis for a phenotype are the capabilities of the analytical tools (Bailey, 1996). Several powerful techniques have been used to determine at which degree the related gene or genes are expressed in the strain exhibiting the phenotype of interest and what kind of changes in genetic basis results in desired phenotypic difference. Functional genomics consists of those techniques which present a better understanding of the complexity of cellular metabolism and mapping of the cellular effects of genetic modifications at the level of DNA, mRNA, proteins, metabolites and fluxes. The techniques used in functional genomics are briefly explained below (Bro and Nielsen, 2004).

- DNA sequencing: the sequenced genomes of two or more organisms are compared, thereby facilitating discovery of differences in genes and in non-protein-coding DNA, such as promoters.
- transcription profiles: DNA array is used for identification of discriminatory genes characteristic of desired physiological states, such as those contributing to high productivity.
- proteome analysis: the simultaneous large-scale analysis of the protein component of an organism. It gives a closer insight to gene function than transcription analysis.
- metabolite profiling: the metabolome consists of all the intracellular metabolites. Analysis of the metabolome might aid inverse metabolic engineering by giving insight into metabolic function of mutated genes in mutants by comparison with a reference strain.
- comparative flux analysis: Analysis of the fluxome (all fluxes in the cell) gives information about which pathways are active and to what extent they are active. By this means, a better understanding of what is occurring inside the cell at a given time is obtained.

1.7. Chromatographic Analysis: High Pressure Liquid Chromatography

In liquid column chromatography, the stationary phase is contained within a narrow tube through which the mobile phase is forced by gravity or under pressure. The components of the mixture to be analyzed are distributed between the mobile phase and stationary phase in varying proportions. Compounds that interact strongly with the stationary phase migrate very slowly with the mobile phase; in contrast, compounds that are weakly retained by the packing material migrate rapidly with the mobile phase. As a consequence of the differences in mobility between the individual components of a mixture, the sample components are separated into discrete bands (or zones) that emerge from the column at specific ‘retention times’. These bands may be identified

qualitatively and/or further analyzed quantitatively using an appropriate detector (http://www.laballiance.com/la_info/support/hplc1.htm).

HPLC consists of high efficiency columns with sophisticated instrumentation performing at increased pressures and flows. Mixture of components is loaded to a HPLC column with a pump at a high pressure and flow rate. Chemical compounds are eluted based on chemical interactions, usually non-covalent nature between analyte and resin. Separated samples come out of the instrument at different times giving specific peaks. (Nelson and Cox, 2005)

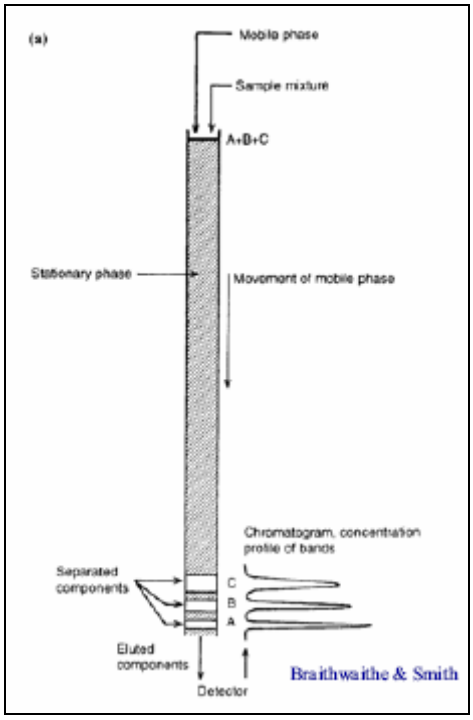

Figure 1.13: Schematic representation of HPLC (http://www.colorado.edu/chemistry/chem5181/C1_Introduction.pdf)

1.8. The aim of the study

The aim of this study was to improve thermotolerance of *F. oxysporum* and its lipase through design and application of evolutionary engineering strategies. Two different evolution strategies were employed and compared with each other with respect to their effectiveness in selecting thermotolerant lipase producers under increasing and constant selective stress conditions. Additionally, the strategies were applied to different morphological states of the organisms: hyphae and spores. Determination of the best selection procedure in terms of physiological state and morphology to which selective stress conditions are applied to was also aimed to be clarified for further evolutionary engineering studies on fungi.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Mould strain

Fusarium oxysporum strain A685 was kindly provided by Prof. Dr. Tajalli Keshavarz, University of Westminster, London, UK. The fungi were maintained on potato-dextrose agar at 4°C and hyphae and spore glycerol (30% v/v) stocks at -80°C.

2.1.2. Mould culture media

2.1.2.1. Composition of solid medium

Commercially available potato dextrose agar (PDA) (Merck) containing potato infusion (4g/L), D-glucose (20g/L) and agar-agar (15g/L) was dissolved in distilled water and autoclaved at 121^{o}C, 1.5 atm15 minutes. PDA plates were preserved at 4^{o}C.

2.1.2.2. Composition of liquid medium

The medium contained basal salts at the following concentrations: 15.6g/L NaH_2PO_4(Merck) , 2g/L KH_2PO_4 (Merck), 330mg/L $CaCl_2.2H_2O$ (Merck), 300mg/L $MgSO_4.7H_2O$ (Riedel-deHaen), 30mg/L $ZnSO_4.7H_2O$(Riedel-de Haen), 12mg/L $MnSO_4.4H_2O$(Riedel-de Haen), 500μg/L $FeSO_4.7H_2O$ (Riedel-de Haen). It was supplemented with 12.5ml/L olive oil (Fluka Biochemica) and 20.5g/L yeast extract (LAB M). The pH of the medium was adjusted to 7.5 with 10M NaOH (Riedel-de Haen). Medium was autoclaved at 121^{o}C, 1.5 atm for 15 min.

2.1.3. Glycerol Stock Solution

100% Glycerol (Carlo Erba Reagent) was mixed with distilled water to a final concentration of 60% (v/v) and autoclaved at 121^{o}C, 1.5 atm. for 15 minutes. It was stored at room temperature.

2.1.4. Spore Harvesting Solutions

Tween-80 (Merck) was dissolved in distilled water to a final concentration of 0.01% (v/v) and autoclaved at 121^{o}C for 15 min. It was stored at room temperature.

2.1.5. Enzyme Assay Buffer and Solutions

Buffer and solutions were freshly made before the assay. Volumes were adjusted depending on sample number.

2.1.5.1. Tris-HCl Buffer

25mM Tris-HCl (Cal Biochemika) was prepared with distilled water and final pH was adjusted to 7.0 with 1M NaOH.

2.1.5.2. Emulsifying Solution

1.1 g/L gum arabic (T.J. Baker) and 2.3g/L sodium deoxycholate (Fluka Biochemika) were dissolved in Tris-HCl buffer pH 7.0

2.1.5.3. Substrate Solution

p-nitrophenyl palmitate(Sigma) was dissolved in acetone(Merck) to a final concentration of 10mM, and then added to emulsifying solution. Final solution was sonicated for 15 minutes for emulsification.

2.1.5.4. Enzyme Reaction Ending Solution

Sodium carbonate (Merck) was dissolved in distilled water to a final concentration of

2M. The solution was stored at room temperature.

2.1.6. Mutagenesis Reaction Ending Solution

10% (w/v) Sodium thiosulphate (Merck) was dissolved in distilled water.

2.1.7. Laboratory Equipments

Autoclave	: NüveOT 4060 Steam Sterilizer (Turkey)
Autoclave	: Tuttnauer Autoclave 2549 ml (Switzerland)
Deep Freezers	: 20 ^{0} C Arçelik (Turkey),-80 ^{0} C Heto Ultrafreeze 4410 (Denmark)
Incubators	: Nüve EN400 (Turkey)
Laminar Flow	: Özge (Turkey)
Microfuge	: Beckman Coulter Microfuge (USA)
Micropipettes	: Ependorf (Switzerland) 1000µl, 200µl 100µl.
pH-meter	: Mettler Toledo MP220 (Switzerland)
Refrigerator	: +4 ^{0}C Arçelik (Turkey)
Sonicator	: B. Braun Lab Sonic® U (Germany)
Thermomixer	: Eppendorf, Thermomixer comfort,1.5-2 ml (Germany)
Ultrapure Water System	: USF-Elga UHQ (USA)
UV-Visible Spectrophotometer	: Shimadzu UV-1601 (Japan)
Water Bath	: Memmert wb-22 (Switzerland)
Refractive index	: Shimadzu RID10A (Japan)
System Controller	: Shimadzu SCL10A (Japan)
Liquid Chromatography	: Shimadzu LC-10AD (Japan)
Degasser:	: Shimadzu DGU-14A (Japan)
Column Oven	: Shimadzu CTO-10AC (Japan)

2.2. Methods

2.2.1. Spore Harvesting

Spores of *F.oxysporum* on PDA slope agar at 4°C were harvested by using sterile glass beads and 0.01% Tween 80. Firstly, 2mm glass beads were added into agar and vortexed. Then 0.01% (w/v) sterile Tween 80 was added to scrape off spores. The resulting suspension was mixed well and spore solution was obtained. The spore concentration in the suspension was determined before spore solution was aliquoted. 500µl spore suspension aliquots were mixed with 500µl 60% (v/v) glycerol and stored in 1.5 ml microfuge tubes containing 30% (v/v) glycerol at -80°C.

2.2.2. Determination of Cellular Dry Weight

Biomass formation was assessed by monitoring changes in dry cell weight. Pre-weighed filter papers (Whatman filter paper no:1) were dried to constant weight at 80°C for 48 hours then allowed to cool to room temperature in dessicator for 30 minutes and weighed (W_1). Then, cultures were filtered under vacuum. Filter papers with wet fungal cells were placed in an oven at 80°C for 24 hours to a constant weight and then weighed (W_2). The cellular dry weight was measured according to Eqn. 2.1:

$$CDW=W_2-W_1 \qquad (2.1)$$

2.2.3. Determination of The Lipase Activity

Lipase activity was assayed on all samples via the colorimetric measurement of p-nitrophenylpalmitate. The substrate (10mM p-nitrophenylpalmitate) was dissolved in acetone then immediately prior to assay, 5 ml of substrate was mixed with 45 ml of emulsifying solution and heated to 37°C with constant stirring. The final solution was sonicated by using B. Braun Sonic for 15 min. 1000 µl of the culture was centrifuged at 14000 rpm for 5 minutes in a Beckman Coulter Microfuge, 100µl of the supernatant was added to 2 ml of substrate and emulsifying mixed solution, vortexed and incubated at 37°C in a shaking water bath for 15 minutes. Hydrolysis reaction was stopped by the addition of 900µl of sodium carbonate. Next, centrifugation was done at 14000rpm for 1

minute in a Beckman Coulter microfuge to remove precipitates. The amount of p-nitrophenyl released was determined by using spectrophotometer readings at 410nm. An assay solution prepared with fresh medium was used as zero reference. The molar extinction coefficient (ε) for this reaction was 15200 $M^{-1}cm^{-1}$. Lipase activity was calculated according to Eqn 2.2:

$$A=\varepsilon.c.l \qquad (2.2)$$

where ε: molar extinction coefficient, c:concentration, l:length

Unit enzyme activity was the amount of enzyme that causes the release of one micromole palmitic acid from one micromole p-nitrophenylpalmitate at 37°C at pH 7.0 for a reaction time of 15 min.

2.2.4. EMS Mutagenesis

One ml of spore suspension (2.10^8 spores /ml) was treated with 20 µl EMS (Sigma) and incubated at 37°C for 1h. The EMS mutagenesis reaction was stopped by adding equal reaction volume of freshly made 10% (w/v) filter sterilized sodium thiosulphate and the mixture was vortexed thoroughly. Mutagenized solution was then washed twice with 0.01 %Tween 80. Spores were collected by centrifugation at 14000 rpm for 10 minutes in Beckman Coulter microfuge and resuspended in 1ml 0.01 % Tween 80. 250 µl of mutant spore solution was inoculated into 50 ml medium and cultivated at 28°C for 7 days. Mutant culture was named as *Foxy 101* and wild type which was not exposed to EMS was named as *Foxy 100*. Mutant spore culture was separated into 250 µl aliquots in 1.5 ml microfuge tubes and stored as spore suspension in glycerol (30% v/v) at -80°C.

2.2.5. Shake Flask Cultivations and Optimization of Sampling Time

Spore suspensions of both mutant and wild type were inoculated into 50 ml of liquid media in 250 ml Erlenmeyer flasks, respectively. The culture was cultivated at 28°C in an orbital shaker at 200 rpm. Optimum sampling time for the highest lipase production was investigated by taking samples aseptically from growing culture in every 24 h. for 7 days. Samples were analyzed for their lipase activities. Cultivations were made in

triplicates.

2.2.6 Thermal Stress Applications as an Evolutionary Engineering Strategy

Two strategies were selected for thermal stress applications .One of which was exposing hyphae and the other one was the exposure of spore suspension to pulse thermal stress.

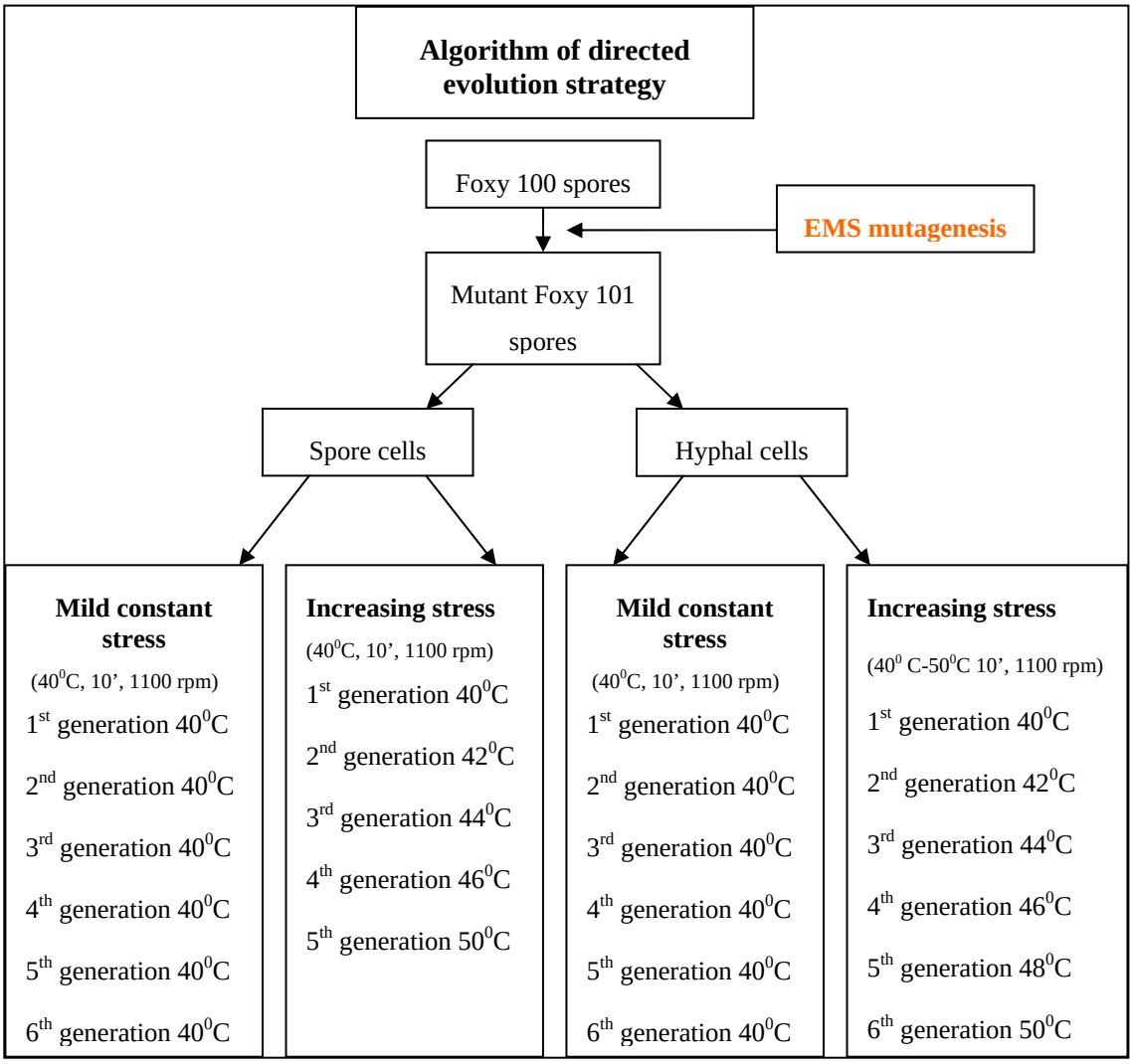

Figure 2.1: Algorithm of directed evolution strategy applied to both morphological state of fungus

Figure 2.1 indicates two different stresses applied to morphological states of fungus in an evolutionary algorithm.

2.2.7 Thermal Stress Applications to Hyphae Inoculum

2.2.7.1. Screening of Heat Shock Responses of Wild Type and Mutant Population

In order to screen thermal response of organisms *Foxy 100* and *Foxy 101*, spore stocks of both organisms were inoculated into 50ml fresh medium and pre-cultured at standard cultivation conditions for 7 days. Then, 1ml was taken from both growing cultures for each heat shock application at different temperatures. Aliquots were exposed to pulse thermal stress at increasing temperatures in a range of 40°C to 60°C for 10 minutes at 1100 rpm, separately. The cells were then collected by centrifugation in a Beckman Coulter microfuge at 14000 rpm for 10 minutes. Supernatants were removed and the pellets were washed twice with fresh medium. Next, the cells were inoculated into fresh medium, and incubated at 28°C at 200 rpm. Samples taken from growing cultures were analyzed to determine lipase activity at around 7^{th} day of cultivation. Controls were obtained in every set of cultivation. Control notations and stress conditions were given in Table 2.1

Table 2.1: Controls used in generation sets obtained from the exposure of hyphae inoculum

Controls		
Control code	Thermal stress exposure time	Stress level (^{0}C)
Foxy100	1	28
FH100D1	1	40
FH100D2	1	42
FH100D3	1	44
FH100D4	1	46
FH100D5	1	48
FH100D6	1	50

2.2.7.2 Directed Evolution of Hyphal Cells to Thermal Stress Levels

Thermal stress was applied in a temperature range of 40°C - 50°C while temperatures were gradually increasing. In the first place, spore suspension was inoculated into 50 ml liquid medium. Inoculation volume was adjusted so as the final concentration was 10^6-10^7 spores in total medium. At 7^{th} day of cultivation, thermal stress was applied to 1 ml of growing culture in a microfuge tube for 10 min. at 1100 rpm in a Thermomixer (Eppendorf). Cells were collected immediately by centrifugation at 14000 rpm for 10 min. in a microfuge (Beckman Coulter). Precipitated cells were then washed with fresh sterile liquid medium twice and resuspended in sterile fresh medium. The suspension was inoculated into 50 ml of liquid medium, incubated at 28°C, 200 rpm. The activities of the cultures and cellular dry weights were measured on the 7^{th} day following inoculation. 20µl of each generation after culturing in liquid medium was directly inoculated onto PDA for sporulation. Higher stress level was applied to the surviving mutant generation according to Table.2.1.a In parallel, constant mild temperature stress at 40°C was applied to the initial EMS-mutagenized population *Foxy 101* for the same number of generation as in the case of increasing stress levels. Generation notations and stress levels were shown in Table 2.1.b

Table 2.1.a: Generations obtained from the exposure of hyphae to increasing stress levels.

Generation code	Generation number	Stress level (^{0}C)
FIH1	1	40
FIH2	2	42
FIH3	3	44
FIH4	4	46
FIH5	5	48
FIH6	6	50

Table 2.1.b: Generations obtained from the exposure of hyphae to constant stress levels.

Generation code	Generation number	Stress level (^{0}C)
FCH1	1	40
FCH2	2	40
FCH3	3	40
FCH4	4	40
FCH5	5	40
FCH6	6	40

2.2.8. Thermal Stress Applications to Spore Inoculum

2.2.8.1. Screening of Heat Shock Responses of Wild Type and Mutant Population spores

In order to understand the response of spores to heat shock, freshly harvested spores suspended in 0.01% Tween 80 with a concentration of 10^6-10^7 spores/ml were centrifuged at 14000rpm for 10 min. Spores were then resuspended into 0.09 % NaCl solution without changing spore concentration. Spore suspensions were directly exposed to thermal stresses simultaneously at different temperatures ranging from 40^0C to 60^0C. Heat-stressed spores were washed twice with 0.09 % NaCl to remove extracellular metabolites and inoculated into 50 ml sterile fresh media. Incubation was done at 28^0C, 200rpm. Growing *Foxy100* and *Foxy101* cultures were terminated on the 7th day of incubation. Biomass formation and lipase production were assessed. In Table 2.2 control sets applied in every spore inoculum experiments.

Table 2.2: Positive controls used for each generation set

Controls		
Control code	Thermal stress exposure time	Stress level (^{0}C)
Foxy100	1	28
FS100D1	1	40
FS100D2	1	42
FS100D3	1	44
FS100D4	1	46
FS100D5	1	50

2.2.8.2. Directed Evolution of Spore Cells to Thermal Stress Levels

Spores suspended in 0.01% Tween 80 with a concentration of 10^6-10^7 spores/ml were centrifuged at 14000 rpm for 10 min. Spores were then resuspended into 0.09 % NaCl solution without changing spore concentration. Afterwards, spore suspension was exposed to thermal stress at gradually increasing temperatures. Heat stressed spores were washed twice with 0.09 % NaCl to remove extracellular metabolites and inoculated into 50 ml sterile fresh medium and incubated. Growing *Foxy100* and *Foxy101* cultures were terminated on the 7th day of incubation. Biomass formation and lipase production were assessed. 20 microliters of grown generation was inoculated onto PDA for sporulation. Spores for the next thermal stress and generation creation were obtained from surviving cells of previous generation. Additionally, constant mild stress at 40^{0}C was also applied to mutant spore population Foxy 101 continually and generations were also formed. Generations obtained from spore inocula were given in Table 2.2.a.and Table 2.2.b

Table 2.2.a: Generations obtained from the exposure of spores to increasing stress levels.

Generation code	Generation number	Stress level (^{0}C)
FIS1	1	40
FIS2	2	42
FIS3	3	44
FIS4	4	46
FIS5	5	50

Table 2.2.b: Generations obtained from the exposure of spores to constant stress levels.

Generation code	Generation number	Stress level (^{0}C)
FCS1	1	40
FCS2	2	40
FCS3	3	40
FCS4	4	40
FCS5	5	40
FCS6	6	40

2.2.9. Extraction of trehalose

The fungus culture was filtered under vacuum and put into 1.5 ml microfuge tubes. Mycelia were suspended into 0.09% NaCl. Spores were harvested from PDA agar with 0.01 % Tween 80 and centrifuged at 14000 rpm for 15 min. Spores were then resuspended in 0.09% NaCl. Generations and their controls were exposed to 40^{0}C heat shock for 10 min. at 1100 rpm using a thermomixer. Samples were obtained from constant stress selection; FCH6, Foxy100 hyphae and FCS6 and Foxy100 spores.

Increasing stress generations, FIH6, FIS5 and Foxy100 hyphae and Foxy100 spores treated with the same procedure explained above were exposed to 50^0C heat shock for 10 min. at 1100 rpm in a thermomixer.

In order to disrupt, mycelial mat and spores were placed into a cold mortar, liquid nitrogen was added and grind to a fine powder with pestle. The powder was suspended into 1 ml distilled water. Before loading to HPLC, samples were filtered through 0.2μm filter. (http://www.ambion.com/techlib/tb/tb_183.html)

2.2.10. HPLC Analysis of Trehalose

The analysis of the trehalose was carried out using HPLC, equipped with an Aminex HPX−87H column. Pure trehalose was used to determine retention time in the column. Trehalose was found to be eluted between 6.89-7.00 min. at 70^0C with a flow rate of 0.6 ml/min. 0.005M H_2SO_4.

3. RESULTS

3.1. EMS Mutagenesis

Genetic diversity was created in wild type *Foxy100* culture by chemically mutagenizing spores with ethyl methane sulfonate (EMS). Mutant population was named as *Foxy101*. After EMS application, mutant and wild type cultures were cultivated. Mutant population showed no significant difference in terms of lipase production and biomass formation. The genetically diverse *Foxy 101* culture was good enough for thermal stress applications.

3.2. Optimization of Sampling Time for Enzyme Activity Measurements

Freshly obtained spores belonging to mutant and wild type cultures were inoculated into the media to a final concentration of 10^6 spores/ml and cultivated. Mutant *Foxy101* and wild type *Foxy100* spores were cultivated for 7 days and lipase productions were monitored. Lipase activity profiles of *Foxy100* and *Foxy101* cultures were shown in Figure 3.1.

There was no lag in lipase production in the early stage of growth. Mutant population had the ability to produce lipase as much as wild type after 4 days following inoculation. Lipase production reaches its maximum level on the 4^{th} day of cultivation and remains almost constant during the following three days. Due to the fact that olive oil was utilized by the organism as the sole carbon source in the media used for cultivation, lipase was produced in parallel with growth. Therefore, lipase production provides indirect information about the growth as well. In parallel to lipase production, *Fusarium oxysporum* enters its stationary phase after 4 days of growth and remains in the stationary phase for the following three days. Heat stress was applied during this phase.

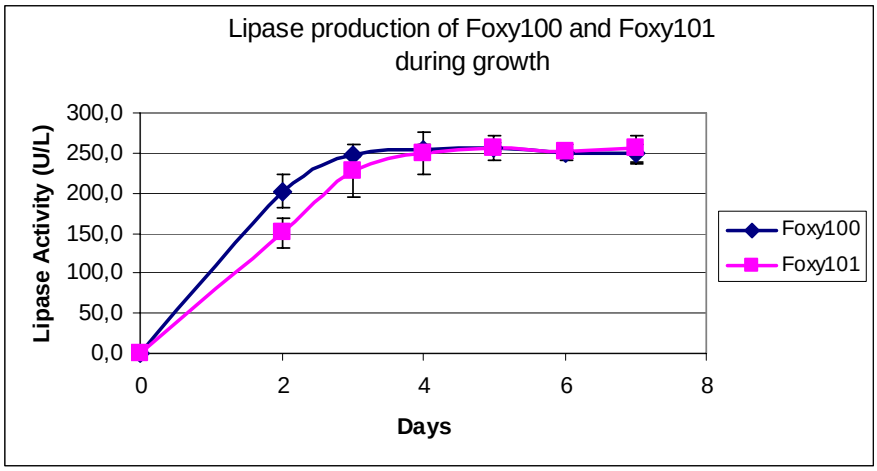

Figure 3.1: Lipase production profiles of wild and mutant cultures during seven days of growth.

Biomass ratio of mutant *Foxy101* is 0.8, based on their final dry weight concentrations relative to the wild type *(Foxy100)* (Fig. 3.2). Although *Foxy101* biomass is less than that of the wild type, lipase activities were comparable.

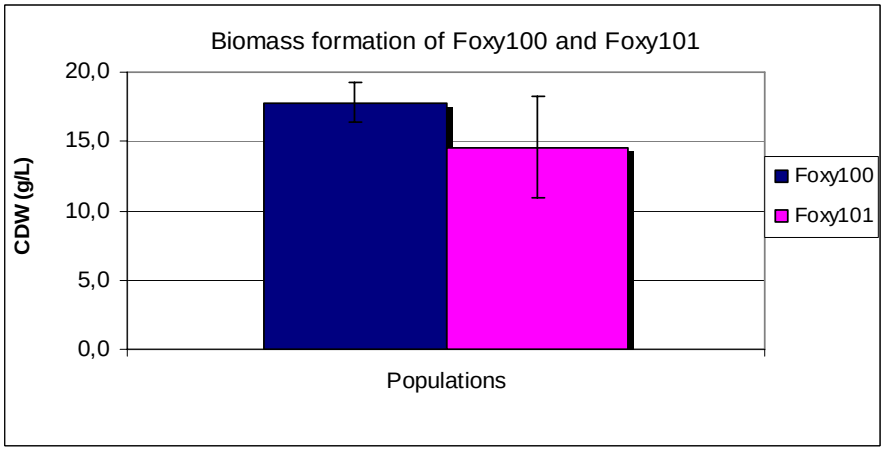

Figure 3.2: Comparison of cellular dry weights of *Foxy100* and *Foxy101* after 7 days of cultivation

3.3. Thermal Stress Applications as an Evolutionary Engineering Strategy

Initially, lowest heat stress level was determined as a sub-lethal, mild temperature to be 40^0C. Two different selection strategies were applied at gradually increasing and constant stress levels at each successive generation of (i) growing hyphae and (ii) spores. The stress levels were increased until no survivors were observed after 7 days of

incubation. In parallel with increasing stress levels strategy, mutants were exposed to the same initial mild stress level for the same number of generations as in the case of selection with increasing stress levels. These sets are denoted as constant heat stress selections.

3.3.1. Thermal stress application on growing hyphae and creation of generations

On the 7th day of pre-cultivation, the hyphae inocula from mutant *Foxy101* and wild type *Foxy100* cultures were exposed to heat shock for 10 min. at 1100 rpm. Positive controls were also generated for each generation.

3.3.1.1 Screening of thermotolerance of growing hyphae of wild type *Foxy100* and mutant *Foxy101*

Growing hyphae inocula were exposed to heat shock according to pre-determined temperatures. Heat stress was applied to each inoculum at a broad temperature range, separately.

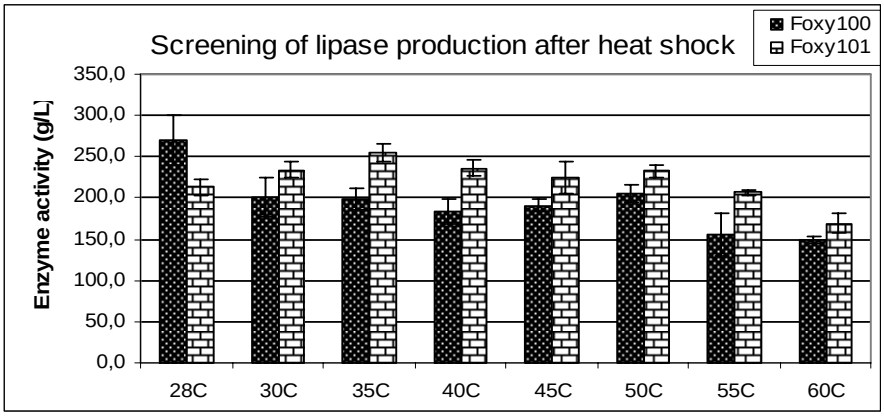

Figure 3.3: Comparison of lipase productions of wild type (Foxy100) and mutant populations (Foxy101) after heat shock applied at different temperatures

Lipase production of wild type *Foxy100* of hyphae inoculants were affected by heat shock treatment. Activities tended to decrease along with increase in temperature. Mixed population *Foxy101* enzyme production showed a different pattern (Fig. 3.3) Lipase production seems to be less affected than that of wild types. Moreover, lipase activity increased around 40^0 C then slightly decreased through 60^0 C but still remained higher than that of wild type values.

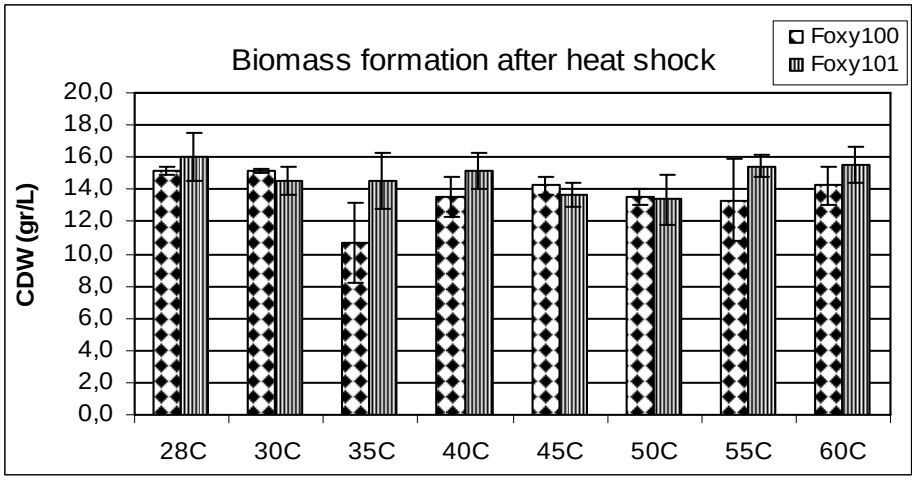

Figure 3.4: Comparison of biomass formation of wild type *(Foxy100)* and mutant populations *(Foxy101)* after heat shock applied at different temperatures

Figure 3.4 shows that heat shock did not significantly affect biomass formation. Wild type and mutant cultures survived the heat shocks at a similar rate as the temperature was increased. Unlike cellular dry weights, however, lipase activities were affected significantly.

3.3.1.2 Overview to activities and biomass formations of six senerations surviving increasing heat stress

Pulse heat stress starting from 40^0C (lowest heat stress level) was applied to hyphae inoculants of *Foxy 101* mixed population by increasing stress level 2^0C at each generation until no lipase activity was observed at 52^0C (FIH7). Lipase activity did decrease over six generations in comparison to screening values at the same temperatures. Thermotolerance of hyphae inoculants did not increase via increasing pulse stress strategy. Generation FIH6 was taken as the last generation. It is seen in Figure 3.5 that generations directed to gain thermotolerance via evolutionary engineering produced similar amounts of lipase with respect to controls.

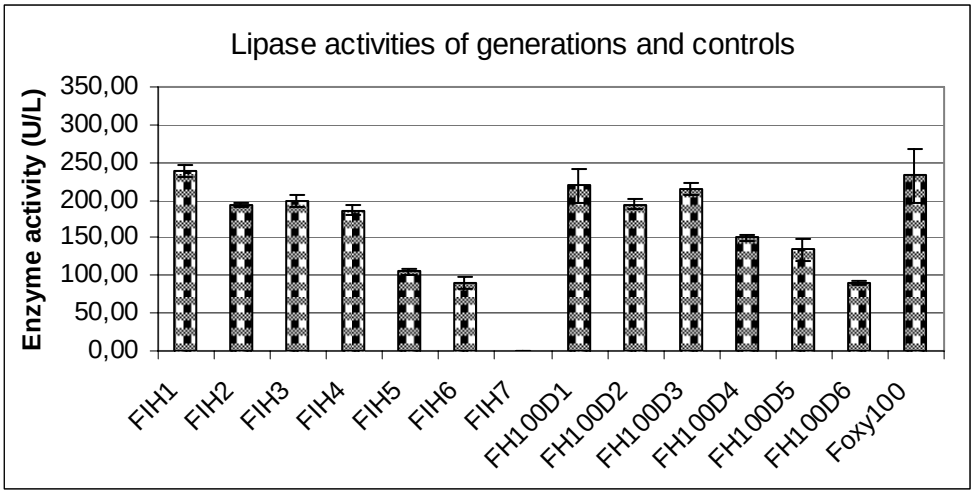

Figure 3.5: Lipase activities of generations (FIHn), controls (FHDn) and wild type *(Foxy100)*

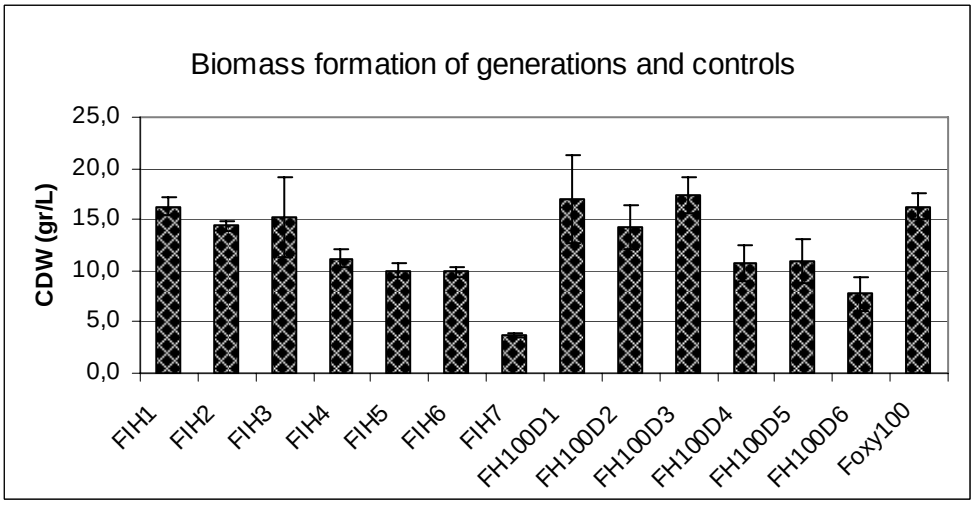

Figure 3.6: Biomass of generations (FIHn), controls (FH100Dn) and wild type *(Foxy 100)*

Likely to decreasing pattern of lipase activities (Fig 3.5), cellular dry weights also decreased (Fig. 3.6). Despite the fact that the 7^{th} generation was able to survive the last heat stress, it did not show any lipase activity. With respect to FH100D6 control, the last generation FIH6 formed higher amounts of biomass.

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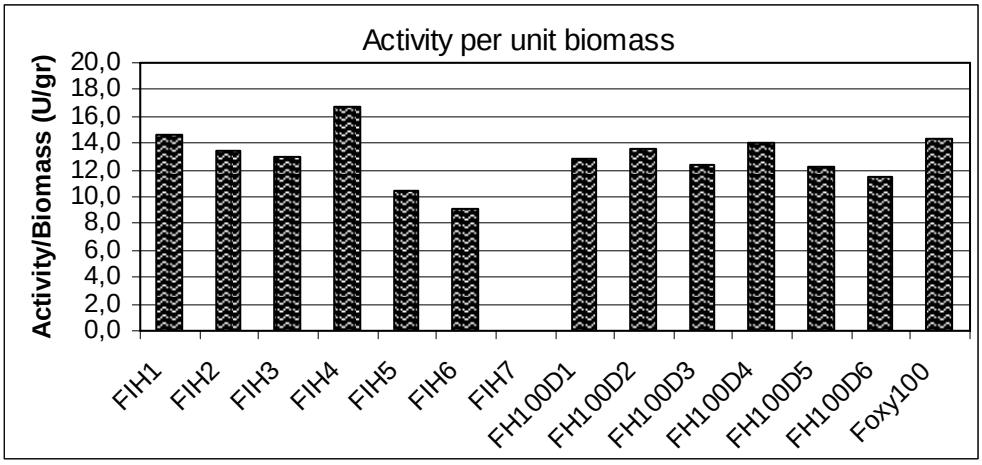

Figure 3.7: Activity per biomass of generations (FIHn), controls (FH100Dn) and wild type *(Foxy 100)*

Lipase production per unit biomass formed value was calculated (Fig.3.7) Lipase production efficiency was decreasing throughout generations. In comparison to controls at corresponding temperatures, generations lipase production was lower and in declining pattern.

3.3.1.3. Overview to activities and biomass formation of six generations surviving constant heat stress

Constant mild stress at 40^0C was applied to hyphae inoculants of mixed population of *Foxy101*. Generations were obtained by applying the same heat stress to survivors of previous generation. Six generations were obtained.

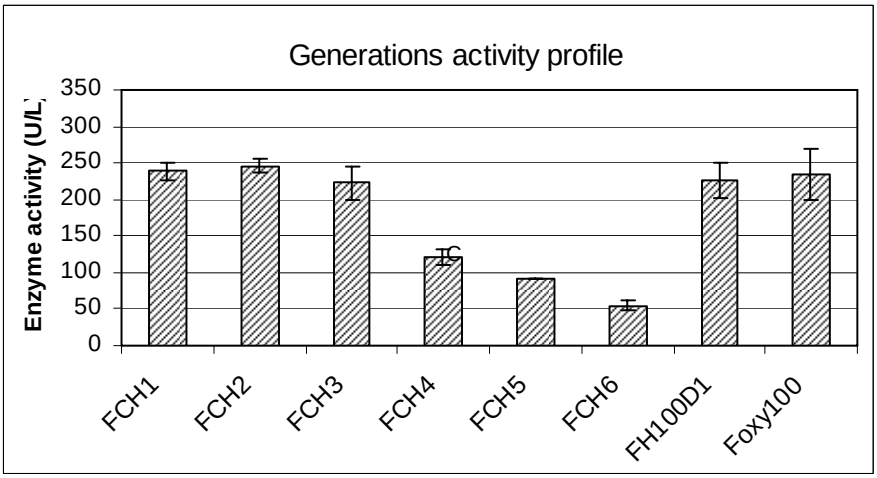

Figure 3.8: Enzyme activity profile of generations after exposure of hyphae to constant heat stress at 40^OC.

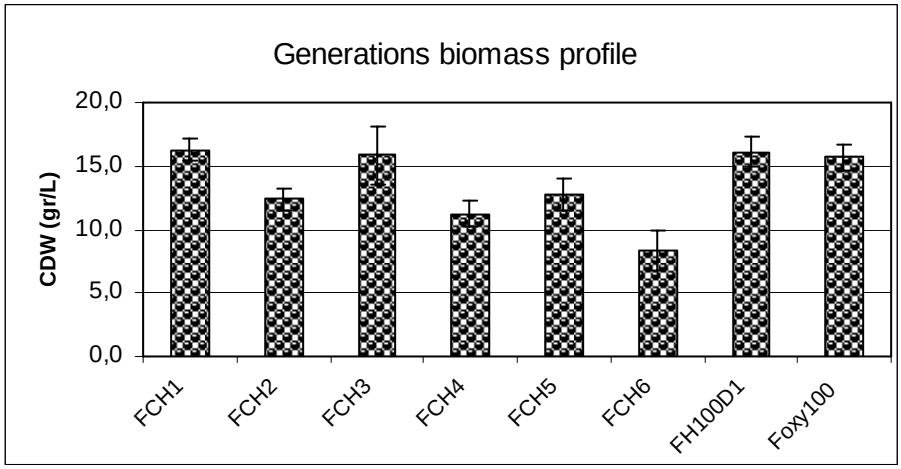

Figure 3.9: Cellular dry weight profile of generations exposed to mild heat stress at 40^{O}C.

Lipase activity was negatively affected by heat stress (Fig.3.8) Last generation's lipase activity was much lower than its corresponding control (FH100D1). Figure 3.9. shows that cellular dry weights measured after cultivation of heat stressed inoculants for seven days. After six generations, FCH6 (last generation that survived 6 times exposure of 40^{0}C) has still the ability to grow and produce lipase.

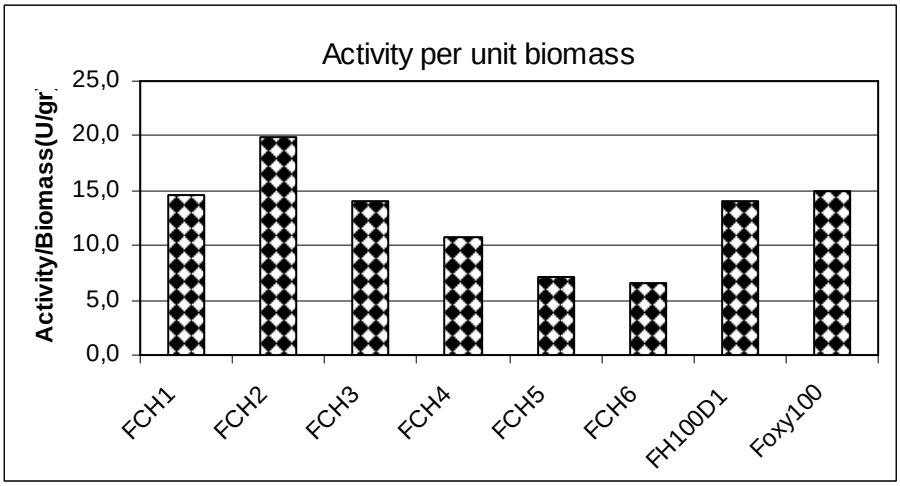

Figure 3.10: Activity per unit biomass of generations exposed to mild heat stress at 40^{O}C.

Lipase produced per unit biomass values were declining along increase in generation number. Max. value among generations belonged to the second generation in

Figure.3.10 which was 20. Last generation's lipase production efficiency was much less than FH100D1.

3.3.2. Thermal stress application on spore suspension and creation of generations

As a second strategy, spore suspensions were exposed to heat stress at similar temperatures.Lipase activity and cellular dry weights of last generations of increasing stress (FIS5) and constant stress (FCS5) were compared to the first strategy in terms of thermotolerance.

3.3.2.1 Screening of thermotolerance of wild type spores

In order to determine thermotolerance limit of wild type spores, they were exposed to similar temperatures as shown in Fig. 3.1. in which range changes between 40^0C-60^0C including the normal growth temperature of the organism ($28\ ^O$C).

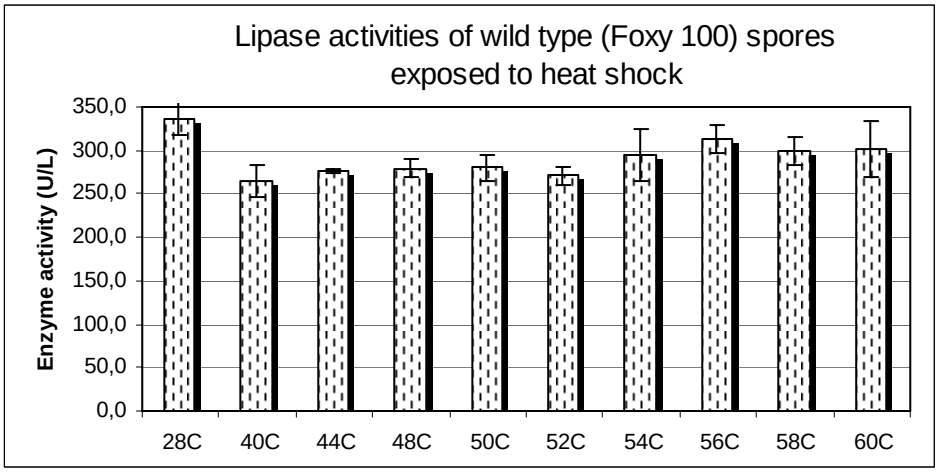

Figure 3.11: Lipase production after exposure of spores to heat stresses at various temperatures.

Figure. 3.11.shows temperatures applied to spores during heat shocks. Wild type spores showed a significant resistance to heat stress at high temperatures. In addition, activities were higher than when hyphae inoculants were exposed to similar temperatures.

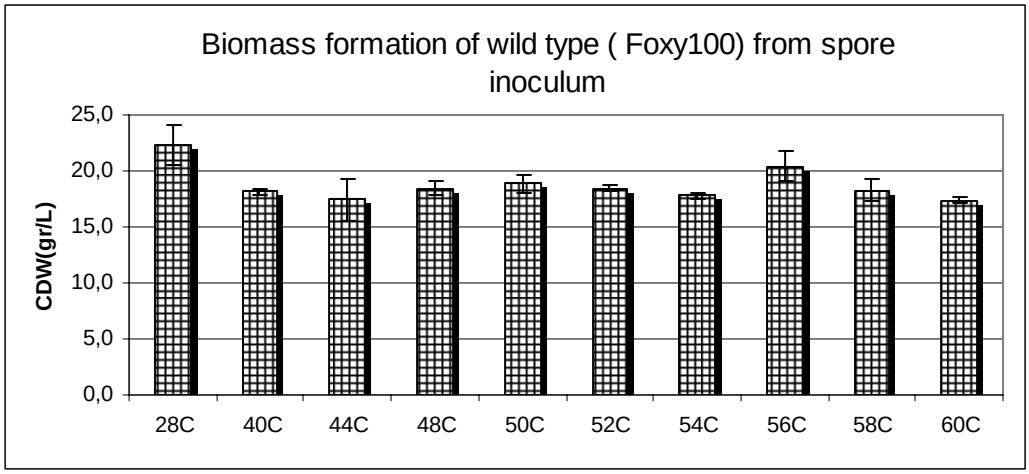

Figure 3.12: Biomass formation of Foxy100 spores after exposure of spores to heat stresses at various temperatures.

Figure 3.12 shows that spores were less affected than hyphae by heat stress at high temperatures. Survival after sudden heat shock was very high even at 60 ^{0}C

3.3.2.2. Overview to activities and biomass formation of six generations surviving increasing stress level

The lowest and the highest heat stresses were applied at 40 ^{O}C and 50 ^{O}C by gradually increasing temperature. Figure 3.13 shows lipase activities of generations and their related controls produced in each set of generation.

Last generation (FIS5) exposed to 50 ^{O}C was able to survive and shows lipase activity. The activity of last generation remained at high levels in comparison to FIH6 generation.

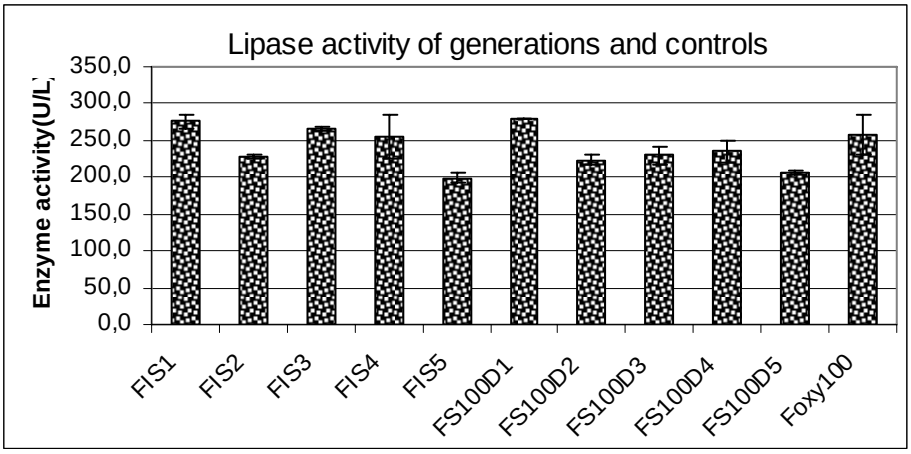

Figure 3.13: Lipase productions of generations after exposure of spores to pulse heat shocks.

Generations' cellular dry weights shown (Fig. 3.14) were higher than the values shown in Fig. 3.6. It could be implied that spores are more tolerant to heat shocks than hyphae of the fungus. Variation in values could be due to the heterogeneity of mutant populations.

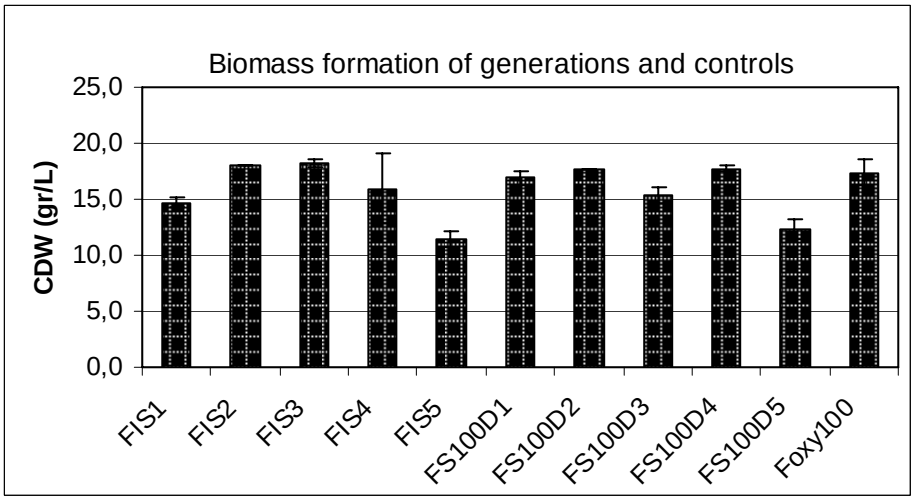

Figure 3.14: Biomass formation of generations after exposure of spores to pulse heat shocks

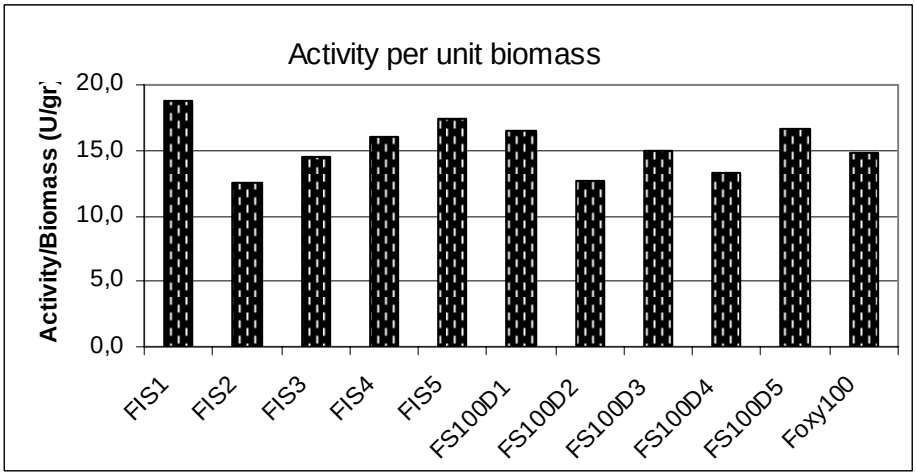

Figure 3.15: Biomass formation of generations after exposure of spores to pulse heat shocks

Figure 3.15 indicates lipase production per unit biomass through 5 generations of spore inocula and their corresponding controls. Generation's response to thermal stress was very good. Mutant population seemed to be directed to resist to high temperatures.

3.3.2.3. Overview to activities and biomass formation of six generations surviving constant heat stress level

Mixed population *Foxy101* was exposed to lowest constant heat stress at 40^0C over five generations. Figure 3.16 shows lipase activities of generations exposed to constant mild stress and their control which was obtained by exposing *Foxy100* to 40^0C once. Lipase activity of last generation (FCS5) was as high as FSD1 (wild type exposed to 40^0C once). Lipase activity was 4-fold of the enzyme produced by FCH5.

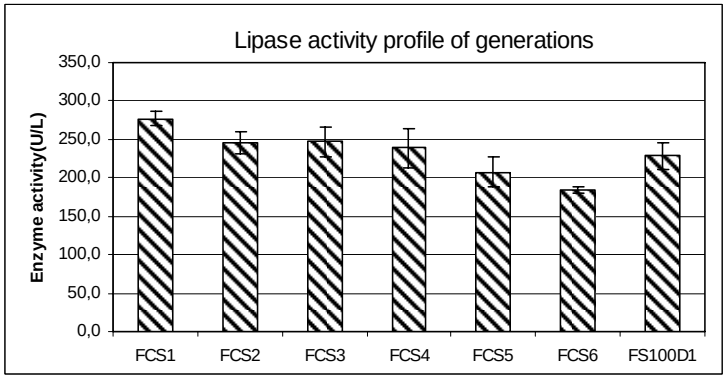

Figure 3.16: Lipase productions of generations after exposure of spores to constant mild heat stress.

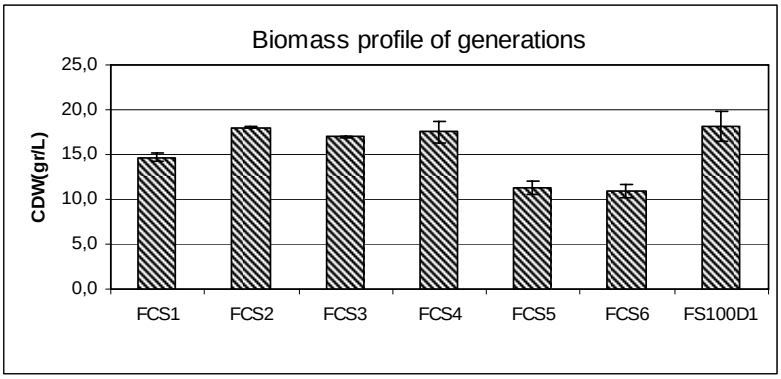

Figure 3.17: Biomass formation of generations after exposure of spores to constant mild heat stress.

Figure 3.17 shows the cellular dry weights belonging to generations and the controls in parallel with lipase production. Cellular dry weight of FCS5 was 1.5 fold higher than FCH5 biomass. All data shows that spores were much more resistant to heat stress than growing hyphae of *Fusarium oxysporum.*

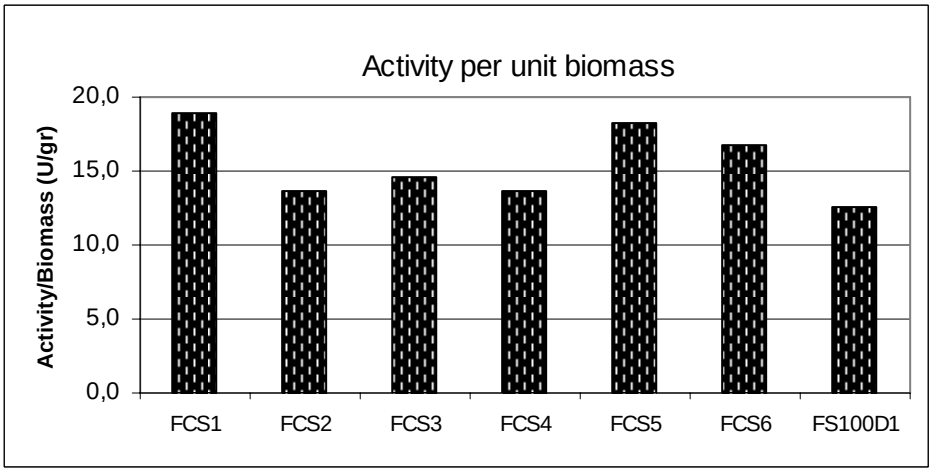

Figure 3.18: Enzyme activity per biomass of generations after exposure of spores to constant mild heat stress

Figure 3.18 shows lipase production of each generation per unit biomass. According to the figure, last generation was able to survive and produce lipase considerably. Its activity per unit biomass value was higher than its control (FS100D1)

3.4. Trehalose Accumulation After Heat Shock

No detectable trehalose peak was observed in any of the sample analyzed. (Data not shown)

4. DISCUSSION AND CONCLUSION

In this study, the primary aim was to improve thermotolerance of *Fusarium oxysporum* and its lipase.The second aim was to investigate the importance of the physiological state of a fungus in terms of thermotolerance during evolutionary engineering studies.

For this purpose, evolutionary engineering, an inverse metabolic engineering approach, was employed. To create genetic diversity prior to evolution, EMS mutagenesis was applied and genetically diverse mutant population was obtained. Directed evolution was then applied to genetically heterogeneous population in the direction of heat stress resistance.

Initial screening of mutant *Foxy101* and wild type *Foxy100* was performed. Lipase production of mutant was similar to wild type during the course of growth for seven days. Directed evolution approach was applied to two physiologically different states of fungus; spore and hyphae, during generation formation. Thermal stress conditions were assessed as gradually increasing selection and constant stress selection strategies.

Initial screening was performed at different temperatures from 28^0C to 60^0C to determine the point that affects lipase production. In both physiological states, lipase activity was not eliminated. Indeed, lipase activity was higher while using spores and spores's did not show a decline along with increase in temperature. In addition, 40^0C was determined as constant mild stress condition and initial stress level in selection strategies. Since there was no sharp decline in biomass at high temperatures, screening should be carried out at higher temperatures where no biomass production or lipase activity is observed.

Evolutionary engineering then applied to hyphae and spore of mutant population. Six generations were obtained from hyphae cultures at two selection strategies by passing the survivors of pulse heat stress step to the following step. After each stress exposure step, the surviving population was randomly selected, and the procedure was continued

with increased stress level in each step. Last generation obtained from the exposure of hyphae inoculants FIH6 was not resistant enough to heat at 50^0C. Although it was able to grow, lipase activity was not observed.

Spores of mutant population were exposed to the same selection procedure starting from 40^0C. Five generations was obtained from increasing stress level strategy ended up at 50^0C as the last temperature. Constant stress condition resulted in six generations.

Lipase production was very close to the value that obtained from direct exposure of *Foxy101* to 50^0C once. In parallel, CDW 's were also showing similar pattern. Variations might due to the existence of heterogenous structure of mutant generations. Constant selection strategy was more effective than increasing stress strategy on spores in spite of the fact that results were not as high as they had been expected. Further generation creation is applicable for obtaining mutant populations having the desired phenotype.

Generations obtained from the exposure of spores to increasing heat stress and constant stress were much more stable and their lipase productions were considerably high even for 50^0C exposure of last generation (FIS5). In terms of cellular dry weight, last generation (FIS5) obtained from exposing spores to heat stress has still the ability to survive and produce lipase with moderate activity Therefore, it was concluded that spores of *F.oxysporum* were more resistant to heat shock under two stress conditions applied. This could be due to either induced trehalose content of spores during heat shock or increased rate of mitosis during sporulation which results in genetic variation. In light of this information, mutant generations were investigated physiologically. Trehalose content of mutant last generations of spore and hypha cultures and wild type were investigated through HPLC. Trehalose accumulation was not found in mutant populations at two physiological states. Thus, another factor other than trehalose should be associated with the increased heat tolerance of spores.

As a result of evolutionary engineering, increasing stress level mutant generations were expected to show higher activity than the *Foxy100* and its controls under the stress conditions. However, last generations from spore and hyphae experiments at two selection strategies were not improved in lipase activity or biomass production.

The applied selection procedure is seemingly not suitable to obtain thermotolerant *F.oxysporum* mutants with high lipase activity. This could be caused by several reasons. First of all, *F.oxysporum* is an organism that can undergo easily. This could have resulted in accumulation of undesired mutations in the genome and crippled strain development.

As a matter of fact, spores responded to thermal stress levels much better than hyphae. Thus, the pulse selection strategy applied on spores in this study could further be continued up to much higher temperature values. (e.g. 60^0C and beyond).Evolutionary engineering approach has been successful on yeast and bacteria over years. However, fungus studies should be improved and optimized in terms of selection parameters. Instead of pulse thermal stress, continuous heat stress would most likely result in better mutant generations. Alternatively, submerged culturing could be replaced by solid state so that spores on solid medium would be forced to survive under stress conditions.

The results imply that selection strategies should be chosen carefully in terms of physiological state in evolutionary engineering applications on fungi.

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RESUME

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